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Effect of Exercise, Diet and Ethnicity on Metabolic Responses in Postprandial State

By

Khlood Jamil Ghafouri

A doctoral Thesis

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**University
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Abstract

Cardiovascular disease is a leading cause of mortality and morbidity worldwide. One of the key factors mediating cardiovascular disease risk, and the underlying atherogenic disease process, is disturbances to metabolism in the postprandial state, particularly with respect to lipoprotein metabolism.

A number of studies have demonstrated that prior exercise can reduce postprandial triglyceride (TG) concentrations, with recent evidence indicating that increased clearance from the circulation of large very low density lipoproteins (VLDL₁) plays an important role. However, it was unclear how exercise facilitated this potentially beneficial effect and this was the focus of the present work.

The first experimental study in this thesis demonstrated, in 10 overweight/obese men, that 90 minutes of prior moderate exercise increased the affinity of VLDL₁ for TG hydrolysis by lipoprotein lipase by 2.2-fold in the fasted state ($p = 0.02$) and 2.6-fold in the postprandial state ($p = 0.001$), but did not significantly alter the affinity of chylomicrons, a novel observation that adds to understanding of the mechanism by which exercise lowers TG concentrations.

Postprandial responses to meal ingestion depend on the macronutrient composition of the food ingested. In the second experimental chapter, postprandial responses to ingestion of a test meal containing 75g glucose, or 75g fat, or a combination of 75g glucose and 75g fat were compared in 10 overweight/obese men. The main finding was that co-ingestion of fat with the glucose load reduced the postprandial glucose response, but not insulin response, compared with glucose ingestion alone. Co-ingestion of fat with the glucose load also substantially reduced the postprandial suppression of non-esterified fatty acids (NEFA) compared to glucose only ingestion. Postprandial TG responses were similar when only fat was consumed compared with co-ingestion of fat and glucose, but postprandial VLDL₁ concentrations were lower in the latter condition.

It is well established that ethnic differences exist in the prevalence of cardio-metabolic diseases. In particular, diabetes prevalence is high in Middle-Eastern

populations. It is not known whether ethnic differences in postprandial metabolism contribute to these differences in risk. In the third experimental study, eight white European men and eight men of Middle-Eastern origin consumed a mixed-meal and postprandial responses were assessed. Postprandial insulin responses were higher in the Middle-Eastern men and postprandial TG concentrations were higher in the European men. This suggests that ethnic differences may exist in the inter-relationship between insulin resistance and lipoprotein metabolism.

Thus, overall this thesis has provided insights into how postprandial metabolism is modulated by exercise, macronutrient intake and ethnicity.

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Wear gratitude like a cloak and it will feed every corner of your life. ~Rumi

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Personnel	Contributions
Prof. Jason Gill	Principal Supervisor
Prof. Muriel J. Caslake	Second supervisor and laboratory assistance.
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“Allah will raise those who
have believed among you and
those who were given
knowledge, by degrees.”

Surat Al-Mujādila-Holy Quran

“If you are irritated by every
rub, how will your mirror be
polished”- Rumi

إِذَا غَاظَرْتَ فِي شَرْفِ مَرُومٍ
فَلَا تَقْنَعُ بِمَا دُونَ النُّجُومِ
فَطَعْمُ الْمَوْتِ فِي أَمْرِ حَقِيرٍ
كَطَعْمِ الْمَوْتِ فِي أَمْرِ عَظِيمٍ

الْمُنْتَبِي

Author's Declaration

Unless otherwise indicated by acknowledgment or reference to published literature, the presented work in this thesis is the author's own and has not been submitted for a degree at another institution.

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The findings of some of the studies have been published as follows:

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Definitions/Abbreviations

4S	The Scandinavian Simvastatin Survival Study
AA	African-American
ABCA1	ATP-binding cassette transporter
AL	Adipose Lipase
ALA	α linolenic acid
AMPK	Activated protein kinase
Apo	Apolipoprotein
ART	Antiretroviral therapy
AUC	Area under curve
BHF	British Heart Foundation
BMI	Body mass index
BMR	Basal metabolic rate
CA	Caucasian
CAD	Coronary artery disease
CCK	Cholecystokinin
CE	Cholesteryl ester
CHD	Coronary heart disease
CETP	cholesteryl ester transfer protein
CHO	Carbohydrate
Co ₂	Carbon dioxide
CM	Chylomicrons
COMB	Trial using combination of fat and glucose
CRL	Cholesterol rich lipoprotein
CRP	C-reactive protein
CT	Computed tomography
CV	Coefficient of variation
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DHA	Docosahexaenoic acid

DNL	<i>de novo</i> lipogenesis
DXA	dual-energy X-ray absorptiometry
EE	Energy expenditure
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
EU	European
Ex	Exercise Trial
FA	Fatty acid
FC	Free cholesterol
FFA	Free fatty acid
FPG	Fasting plasma glucose
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
HBA _{1c}	Hemoglobin A _{1c}
HDL	High Density Lipoprotein
HDL-C	High Density Lipoprotein cholesterol
HL	Hepatic lipase
HOMA _{IR}	Homeostasis model of assessment - insulin resistance
HPS	The Heart Protection Study
HSL	Hormone-sensitive lipase
HSPG	Heparin sulphate-proteoglycans
IDF	International Diabetes Federation
IDL	Intermediate density lipoprotein
IAF	Intra-Abdominal Fat
IGT	Impaired glucose tolerance
IHD	Ischemic heart disease
INTERHEART	The international heart study
IOTF	International Obesity Task Force
IR	Insulin resistance
kDa	Kilo Dalton

LCAT	Lecithin cholesterol acyltransferase
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein Cholesterol
LDLR	LDL receptor
LGI	Low glycaemic index diet
LRP	LDL receptor-related protein
LXRα	liver X receptor- α
LPL	Lipoprotein lipase
ME	Middle Eastern
MENA	The Middle East and North Africa
MetS	Metabolic syndrome
MI	Myocardial infarction
MRI	Magnetic resonance imaging
MUFA	Monounsaturated fatty
MW	Molecular weight
n-3 PUFA	omega 3 polyunsaturated fatty acids
NaOH	Sodium hydroxide
NEFA	Non-esterified fatty acids
NCEP-ATP III	National Cholesterol Education Program Adult Treatment Panel
NHB	non-Hispanic Black
NHS	National Health Services
NHW	Non- Hispanics White
NIDDM	Non-insulin dependent diabetes mellitus
NMR	Nuclear Magnetic Resonance
OFTT	Oral fat tolerance test
OGTT	Oral glucose tolerance test
PDHS	Penn Diabetes Heart Study
pH	Power of hydrogen
PL	Phospholipid
PLTP	phospholipid transfer protein
PUFA	Polyunsaturated Fatty Acid

PVD	Peripheral vascular disease
QC	Quality control
RCT	Reverse Cholesterol Transport
RCTs	Randomized control trials
RER	Respiratory exchange ration
RLPs	Remnant-like lipoprotein particles
RPE	Ratings of perceived exertion
SA	South Asian
SBP	Systolic blood pressure
SCRIP	Stanford Coronary Risk Intervention Project
SD	Standard deviation
sdLDL	Small dense low density lipoprotein
SEM	Standard error of the mean
SER	sterol response elements
SES	Socioeconomic status
S _f	Svedberg floatation rate
SFA	Saturated fatty acid
SR-BI	scavenger receptor B1
STPD	Slandered temperature and pressure
T2D	Type-2 diabetes
TAUC	Time-averaged area under curve
TC	Total cholesterol
TFA	Trans fatty acids
TG	Triglyceride
Tris-HCl	Trisaminomethane hydrochloride
TRL	Triglyceride rich lipoprotein
VLDL ₁	Very low density lipoprotein 1
VLDL ₂	Very low density lipoprotein 2
VO _{2max}	Maximal oxygen consumption
WC	Waist circumference
WOSCOP	West of Scotland coranary prevention study

WHO

WHR

World Health Organisation

Waist-hip ratio

1 Introduction and literature review

1.1 Introduction

Cardiovascular disease (CVD) is a family of common multifactorial diseases, including coronary heart disease (CHD), cerebrovascular disease, hypertension, and heart failure, which develop as a consequence of interactions between the innate factors, encoded in a person's genotype interacting with environmental factors (e.g. nutrition, smoking, inactive lifestyle) (Sing et al., 2003). Atherosclerosis, which develops over decades, is the underlying pathology of these conditions (Griffin, 1999). Cardiovascular disease, together with related metabolic diseases, such as diabetes - often termed cardio-metabolic diseases - are leading causes of disability and premature death around the world, and make a substantial contribution to total health care costs (WHO, 2014a). According to the World Health Organization (WHO) an estimated 16.7 million - or 29.2% of deaths - result from the various forms of CVD (WHO, 2014a). In the UK, CVD was a major cause of death, accounting for almost 155,000 deaths in 2014 (Townsend et al., 2015). Thus, this disease places an extensive economic burden on healthcare systems (Deaton et al., 2011, WHO, 2014a). More than £6.8 billion was spent on treating CVD within the NHS in England in 2012/2013 (British Heart Foundation, 2012). The highest expenditure was on secondary care with £4373 million spent on secondary care for CVD in England. Within secondary care, emergency admissions had the greatest expenditure. Within primary care, the second highest setting for expenditure, the majority of costs were due to prescribing (£1387.5 million). In 2012/2013 in Wales, a total of £442.3 million was spent on CVD, in Northern Ireland, £393 million was spent and in Scotland it is estimated that >£750 million was spent on treatment of CVD (Bhatnagar et al., 2015).

Environmental factors influencing CVD can be classified as non-modifiable risk factors, such as age, sex, family history and ethnicity, and modifiable risk factors (see Figure 1.1). Much of our knowledge about the latter comes from the pioneering Framingham study (D'Agostino Sr et al., 2001). This longitudinal cohort study

identified a number of potentially modifiable risk factors including high levels of cholesterol and triglycerides, hypertension, diabetes, high adiposity, obesity, smoking, unhealthy diet, and lack of physical activity (WHO, 2014b, Bitton and Gaziano, 2010). Although many cases of CVD are potentially preventable by action on risk factors such as unhealthy diet, physical inactivity and smoking, the public health and economic importance of CVD means the need to fully understand all the root causes of CVD prevalence remains.

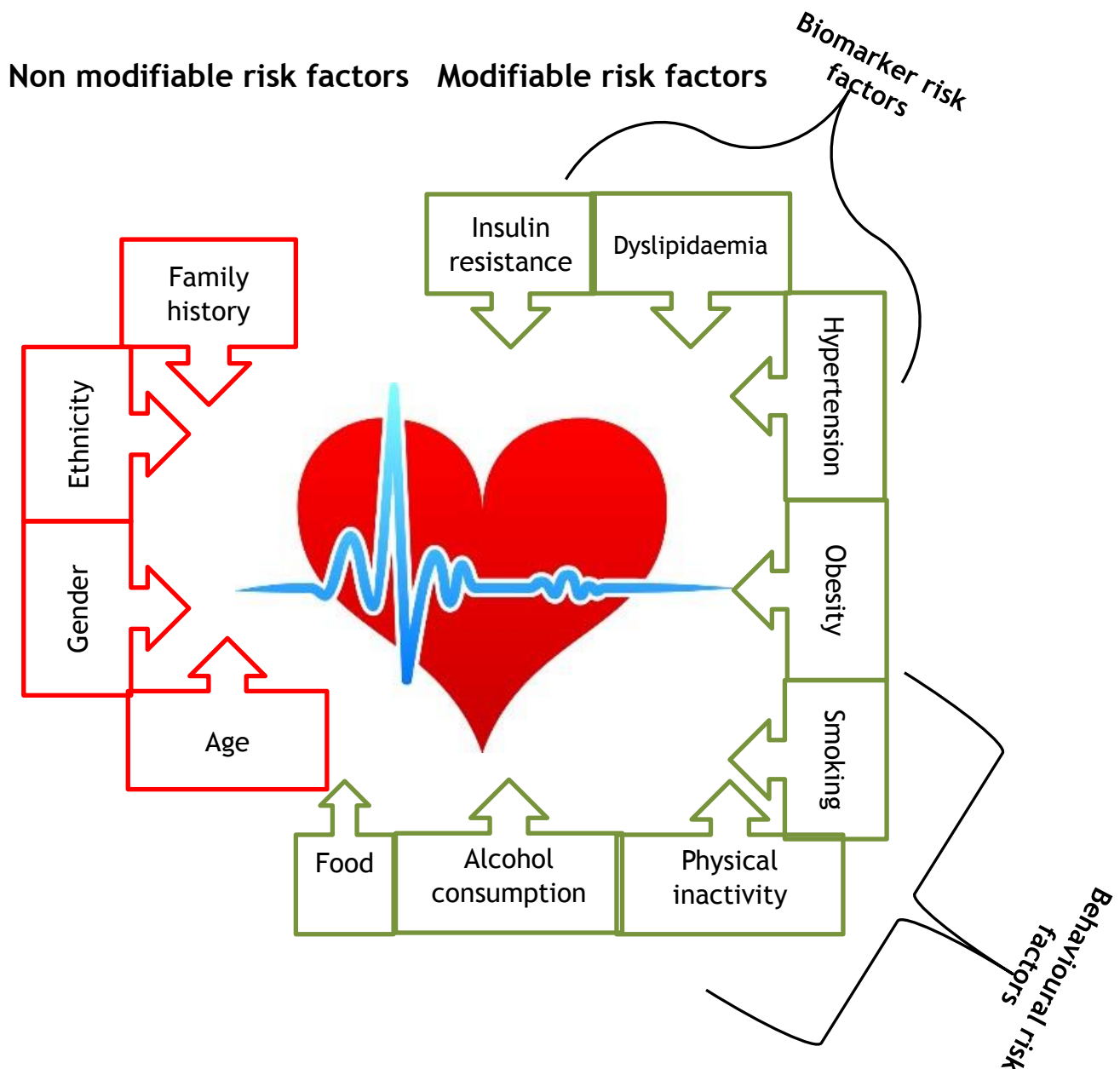


Figure 1.1. Cardiovascular diseases risk factors.

1.2 Non-modifiable risk factors

Age, sex, family history and ethnicity are factors that cannot be modified. Age plays an important role in the development of cardiovascular disease. The risk of CVD increases approximately by 3 fold with each decade of life (Finegold et al., 2013). Similarly, the risk of having a stroke doubles every decade after the age of 55 (American Heart Association, 2011, Brown et al., 1996, Wolf et al., 1992). Even in developed countries, despite a decrease in mortality due to CVD, mortality from CVD still increases with age (Finegold et al., 2013). Approximately 82% of CVD deaths occur after 55 years of age (Mackay and Mensah, 2004). The reasons for the increase in incidence of CVD with age are not completely clear, however, with advancing age the serum total cholesterol concentration increases which is likely to contribute to the effect (Jousilahti et al., 1999). Loss of compliance and functions of blood vessels due to mechanical and structural changes in vessel wall with advancing age, may also contribute to CVD development and progression (Jani and Rajkumar, 2006).

Sex contributes to 40% of the variation in coronary heart disease mortality (Jousilahti et al., 1999, Hu and Group, 2003). Epidemiologically, the sex difference in prognosis of CVD suggests an intrinsic sexual difference in susceptibility to CVD (Finegold et al., 2013, Kannel et al., 1976, WHO, 2014a). In developed countries, CHD is being two to five times more common in men than in women in the younger age groups. (Möller-Leimkühler, 2007). CHD risk increases with age in both men and women, but shows a more prominent increase in women older than 50. Despite better medical treatment of CHD, It remains the leading killer of women (Mosca et al., 2007). In Europe, about 55% of all female deaths are caused by cardiovascular disease (CVD), especially CHD and stroke, compared with 44% of all male deaths (Petersen et al., 2005). Age-adjusted mortality for CVD has continuously declined in the last four decades, but to a lesser extent in women than in men. In fact, the temporal trend of the incidence of CVD even shows a rise in women (Tunstall-Pedoe et al., 1999, Thom et al., 2006). This has been mainly attributed to a decrease in myocardial infarction incidence in younger men, with a concomitant increase in older women (Tunstall-

Pedoe et al., 1999). Although, the mechanism behind these differences is unclear, some explanations such as hormonal difference in premenopausal stages were suggested. Oestrogen, for example may provide protective effects through glucose metabolism and haemostatic system indirectly and may improve endothelial cell function directly (Jousilahti et al., 1999).

Family history of CVD is another unmodifiable risk factor that is an independent predictor of future disease incidence (Hunt et al., 1986). The risk of developing CVD doubles if a first degree male relative develops coronary heart disease or stroke before the age of 60 or if a first degree female relative does so before 65 years (British Heart Foundation, 2012, Sesso et al., 2001). The risk increase by 50%, if both parents have suffered from heart disease before the age of 55 (British Heart Foundation, 2012).

Finally, ethnic origin can play a role in the incidence of CVD. There is ample evidence for substantial and persistent disparities between racial/ethnic groups in cardiovascular disease and associated risk factors, such as obesity, hypertension and diabetes (Mensah and Brown, 2007, Halder et al., 2012). Research into ethnic differences in general health has mainly focused on factors that are related to the ethnic minorities directly, such as genetic, socioeconomic, and sociocultural factors (Venema et al., 1995). Another possible determinant of ethnic health differences is health care itself, more specifically the accessibility and the quality of health care (Venema et al., 1995, Lanting et al., 2005). However, it has been reported that South Asian men and women in the UK experience approximately 50% higher age-standardised CHD mortality than European Whites (Wild et al., 2006). Also, compared to Caucasians, African-Caribbeans and people of African descent have high incidence of stroke (Balarajan, 1991, Cappuccio, 1997), end-stage renal failure but lower CHD (Roderick et al., 1994, Raleigh, 1997). This may be due to higher rates of high blood pressure: in those of African ancestry, hypertension is three- to four- fold more prevalent than in Caucasians (McKeigue et al., 1991, Cruickshank et al., 1991, Cappuccio et al., 1997, Joffe et al., 1992, Chaturvedi et al., 1994), as is diabetes

(Shai et al., 2006, McKeigue et al., 1991, Simmons et al., 1991). Risk factors in South Asians are hyperinsulinaemia, raised TG and low HDL cholesterol levels, central adiposity with a high waist to hip ratio (McKeigue, 1991, McKeigue et al., 1992, Cruickshank et al., 1991). Whereas in Africans with glucose intolerance there is a low triglyceride and high HDL cholesterol levels, due to lower visceral adiposity when compare to whites at the same BMI (Chaturvedi, 1993, Chaturvedi, 1994, Cooper et al., 1997, Cappuccio, 1997).

1.3 Modifiable risk factors

The international heart study over 52 countries (INTERHEART) assessed the importance of risk factors for coronary artery disease worldwide (Yusuf et al., 2004a, Yusuf et al., 2004b). Nine measured and potentially modifiable risk factors, accounted for more than 90% of the proportion of the risk for acute myocardial infarction. These can be classified into two categories, modifiable biomarkers and modifiable behavioural factors. The modifiable markers are linked to metabolic syndrome (MetS). Metabolic syndrome had been identified by Dr. Gerald Reaven almost three decades ago. Originally known as “syndrome X”, the cluster has also been termed pluri-metabolic syndrome (MS), or Reaven's syndrome. These characteristics, when found in the same person, are so ominous that they have also been called “the deadly quartet” or “the awesome foursome”(Reaven, 1988). Reaven and subsequently others postulated that insulin resistance underlies Syndrome X (hence the commonly used term insulin resistance syndrome) (Haffner et al., 1992). According to the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) panel, MetS include an increased waist circumference, blood pressure elevation, low HDL cholesterol, high triglycerides, and hyperglycaemia (NCEP, 2002). Although the risk associated with the MetS is well-documented, the definition of the syndrome is still in flux. In addition, there are behavioural risk factors include, physical activity, diet pattern, smoking, psychosocial factors and alcohol consumption. The effect of these risk factors was consistent in men and women across different geographic regions and by ethnic group. The British Regional Heart Study

also found that smoking, blood pressure and cholesterol accounted for 90% of attributable risk of CVD (Emberson et al., 2003). CVD can be prevented through modification of behavioural risk factors and /or through pharmacological therapy for medical conditions (e.g., using blood pressure-lowering agents and lipid-modifying agents). Modifiable and non-modifiable risk factors can interact with each other's, thus, through a combination of treatments and healthy lifestyle choices, the risk of heart disease and stroke can be significantly decreased (See **Figure 1.2**).

1.3.1 Behavioural factors

Socioeconomic status and psychosocial factors

Socioeconomic status (SES) refers to an individual's social position relative to other members of a society. A number of studies suggest that poor living conditions in childhood and adolescence contribute to increased risk of developing CVD in both genders (Clark et al., 2009, Kaplan and Keil, 1993). The social status is linked also to alcohol consumption. The Marmot Review (2010) mentioned an association between alcohol-related hospital admissions and high levels of deprivation for both men and women, with particularly high rates of admission for those areas among the most deprived quintile of England and Wales. Binge drinking was also reported to be most common among those living in deprived areas (Marmot, 2010). Cigarette smoking follows a social class gradient in the USA and most developed countries. Starting in youth, smoking initiation is positively correlated with being from a low income household and performing poorly in school (Barbeau et al., 2004b, Elders, 1997, Steptoe et al., 2002). The prevalence of smoking is linked to low educational attainment (Pierce et al., 1989, Cavelaars et al., 2000, Barbeau et al., 2004a), working class occupations (Bang and Kim, 2001, Barbeau et al., 2004a), and lower income levels (Barbeau et al., 2004a, Control and Prevention, 2002, H., 2005). Thus, cigarette smoking is clearly associated with social disadvantage as defined by educational attainment, income, and occupational class. Cigarette smoking is the social behaviour that has the single largest impact on health inequalities. All these risk factors increase the incidence of developing CVD. Moreover, depression and depressive symptoms are common in patients with CVD (Timberlake et al., 1997) or heart failure (Rutledge et al., 2006) and are associated with adverse outcomes (Van Melle et al., 2004) showing strong and consistent evidence that depression and social isolation or lack of quality social support are independent risk factors for the development of and prognosis with CHD (Carney and Freedland, 2016, Valtorta et al., 2016).

Alcohol consumption

Alcohol is one of the behavioural risk factors. It is known to have both beneficial and harmful effects on the biochemical basis for CHD and the psychological consequences of the disease (Foppa et al., 2002). Systematic reviews and meta-analyses have addressed the association of alcohol consumption with cardiovascular disease outcomes (Cleophas, 1999, Corrao et al., 2000, Corrao et al., 2004, Di Castelnuovo et al., 2002, Fillmore et al., 2007, Maclure, 1993, Reynolds et al., 2003). In Scotland, 32% of men and 4% of women drink above weekly recommended limits. Patterns of drinking vary and 44% of men who had drunk in the last week consumed eight units or more on their heaviest drinking day (where one unit is defined as approximately 8 g /10 ml of alcohol), indicating that binge drinking may be a particular problem (Wilson et al., 2015). Drinking is harmful and associated with a poorer lipid profile, and adverse effect on systolic blood pressure and increased risk of thrombosis (McKee and Britton, 1998, Britton and McKee, 2000). Clearly, ethanol consumed with a meal elevates plasma and VLDL-TG (Lee et al., 2005). It has been observed that, the addition of 47.5 g alcohol to a high-fat meal (54 % of energy) was associated with an approximately 60 % increase in the peak plasma TG concentration compared with a meal consumed without alcohol (Fielding et al., 2000). Ethanol has also been shown to increase fatty acid synthesis (Siler et al., 1998) and also to reduce TG clearance from the plasma (Pownall et al., 1999). The relationship between alcohol consumption and stroke is believed to involve various mechanisms including alcohol-induced hypertension, cardiomyopathy, coagulation disorders, atrial fibrillation, and reductions in cerebral blood flow (Malarcher et al., 2001, Hillborn, 1998, Gorelick et al., 1999, Zakhari, 1997). A possible explanation of a reduced risk of ischemic stroke with moderate alcohol consumption is that alcohol increases high-density lipoprotein cholesterol levels and decreases platelet aggregation and fibrinolytic activity (Gorelick et al., 1999, Zakhari, 1997, Stampfer et al., 1988). There does not appear to be any differential effect associated with type of alcohol consumed (Whelan et al., 2004, Di Castelnuovo et al., 2002).

Smoking

Smoking is a strong independent risk factor of cardiovascular events and mortality even at older age, advancing cardiovascular mortality by more than five years (Mons et al., 2015, Gellert et al., 2013, Kenfield et al., 2010). Smoking is linked to a two- to four-fold increased risk of CHD (Go et al., 2014, Kondo et al., 2011). Smoking independently contributes to the risk of myocardial infarction (Ferrie et al., 2009, Teo et al., 2006), stroke (Kelly-Hayes, 2010). A dose-response relationship between the number of cigarettes smoked per day and both myocardial infarction and stroke has been established in large cohort studies (Kenfield et al., 2010, Kondo et al., 2011). It also, influences the risk factor of developing CVD. Cigarette smoking also increases postprandial triglyceride levels by 50% (Pourmand et al., 2004, Axelsen et al., 1995, Chioloro et al., 2008), and studies of young adults have identified early use of alcohol and tobacco as key determinants of subsequent levels of serum triglyceride (Croft et al., 1987), lower HDL-C (Ellison et al., 2004). Data obtained in a large sample of men and women support the interpretation of Axelson et al. that smoking affects postprandial TG metabolism primarily by raising lipoproteins of intestinal origin because cigarette smokers had substantially greater postprandial retinyl palmitate and apo B-48 (by 114 - 259 %) responses than did non-smokers, when adjusted for fasting TG. In addition, smoking may increase insulin resistance directly (Benowitz, 2003, Houston et al., 2006, Janzon et al., 1983). The response of insulin in smokers to an oral glucose test was more pronounced compared to non-smokers (Facchini et al., 1992), insulin resistance was dose dependently related to smoking (Eliasson et al., 1994). Most of the excess risk and risk advancement disappeared within 5 years after smoking cessation. Smoking cessation is highly and rapidly beneficial also at advanced age (Gellert et al., 2013, Iso et al., 2005). A Japanese cohort study found that quitting smoking more than 4 years ago was found to reduce the risk for major cardiovascular events by 70% compared to continuing smoking (Kondo et al., 2011). A systematic review of 20 studies concluded that quitting smoking is associated with a 36% reduction in the relative risk of mortality for patients with CHD who quit compared with those who continued smoking (RR 0.64; 95% CI 0.58 to 0.71). This risk

reduction appears to be consistent regardless of age, sex, index cardiac event, country, and year of study commencement (Critchley and Capewell, 2003).

1.3.2 Biomarker Risk factors

Blood pressure

High blood pressure or hypertension refers to a chronic condition characterised by increase of blood pressure in the arteries. This increase could either be in systolic blood pressure (SBP) which represents the peak pressure due to ventricular contraction during systole, or a diastolic blood pressure (DBP) which represents the pressure during ventricular relaxation in diastole, or both. A reading of ≥ 140 mm Hg of SBP or ≥ 90 mm Hg of DBP indicates the presence of hypertension (Tajeu et al., 2017, Schiffrin et al., 2016).

The Department of Health survey of 2010 for England showed 31.5% prevalence of hypertension in male adults over 16 years and above and 29.0% in women. This puts a huge burden of approximately £2 billion on NHS annually (Public Health England, 2014, British Hypertension Society, 2015). Hypertension is considered as a major risk factor for stroke, myocardial infarction, heart failure, chronic kidney disease, peripheral vascular disease, cognitive decline and premature death (Freis, 1969, Baudouin-Legros and Meyer, 1990, Rapsomaniki et al., 2014). Hypertension affects blood vessels by altering their functionality. It can lead to atherosclerosis and narrowing of the blood vessels making them more likely to block from blood clots or bits of fatty material breaking off from the lining of the blood vessel wall. Both SBP and DBP can be used as predictors of cardiovascular risk (Stamler et al., 1993a), atrial fibrillation (Kannel et al., 1982), coronary heart disease, stroke, transient ischemic attack, and congestive heart failure (Hubert et al., 1983). Untreated hypertension is associated with a progressive rise in blood pressure and shows a strong positive correlation with the risk of CVD onset and mortality (Cutler, 1996). The risk of cardiovascular events doubles for every 20/10 mmHg rise in blood pressure beyond the normal range (Rapsomaniki et al., 2014, Bauchner et al., 2014).

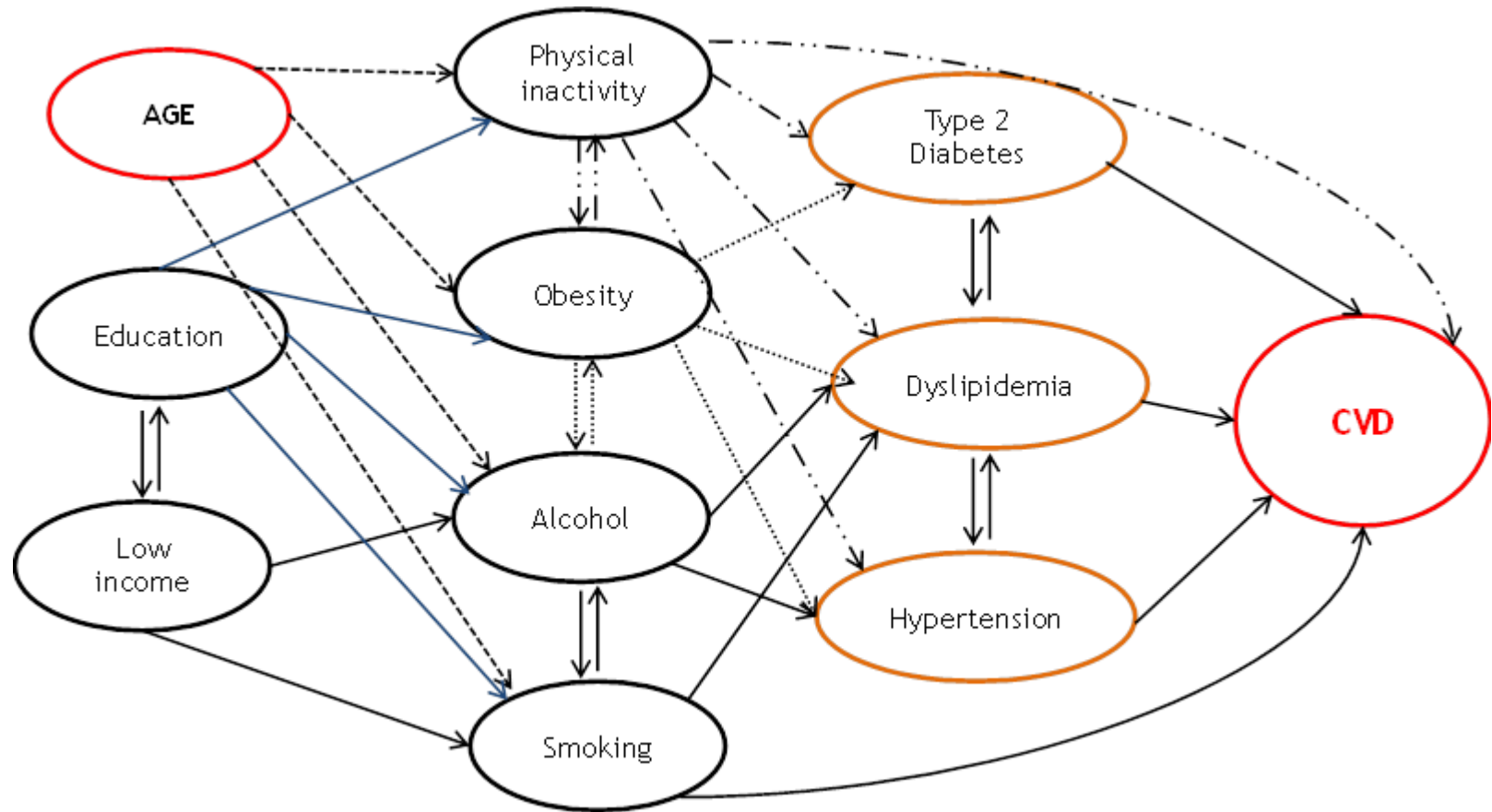


Figure 1.2. Interaction between modifiable risk factors in aged population. Ageing increases negative behaviours like smoking and drinking alcohol. It also, reduces the physical activity for the individuals all these factors contribute to increase body weight and develop some CVD risk factors such as diabetes, dyslipidemia and hypertension.

Lipid and CHD risk

Lipid metabolism disturbance can lead to CVD. These diseases could be a result of inherited defects in lipoprotein metabolism, such as hyper- or hypo-lipoproteinemias, or of a metabolic disorder; abnormal lipoprotein metabolism is often observed as a secondary effect of diabetes, hypothyroidism and kidney disease, or it could be due to life style.

1.4 Lipid metabolism

1.4.1 Lipoproteins

Lipids are hydrophobic and mostly insoluble in blood, so they require transport within hydrophilic, spherical structures called lipoproteins. Circulating lipoproteins are originally produced either from the intestine (exogenous) or from the liver (endogenous). They can be broadly divided in order of increasing density into: chylomicrons (CM), very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). The density of lipoprotein depends on cholesterol, TG and protein content as the more cholesterol and TG and less protein the less dense it is. LDL are the major carriers of cholesterol in blood and contain one molecule of apolipoprotein B100 which is recognized by the LDL receptors (Gurr et al., 2002). VLDL and CM are the major carriers for TG. Lipoproteins can be classified according to their lipid contents into TG-Rich Lipoprotein (TRL; includes CM and VLDL) and Cholesterol Rich Lipoprotein (CRL; includes LDL and HDL (Sparks JD, 1994)) (See Table 1.1).

Table 1.1; Lipoprotein composition (Sparks JD, 1994).

	CM	VLDL	LDL	HDL
Density (g.mL ⁻¹)	<0.96	0.96-1.006	1.006-1.063	<1.063-1.21
Diameter (nm)	100-1000	30-90	20-25	10-20
Apolipoprotein	A,C,E,B48	A,C,E,B100	B100	A,C,D,E
Composition (%)				
Proteins	2	10	20	40
Lipids	98	90	80	60
Lipid composition (%)				
Triglyceride	88	55	12	12
Cholesterol Ester + cholesterol	4	24	59	40
Phospholipid	8	20	28	47
Free fatty acid	-	1	1	1

1.4.2 Apolipoproteins

Apolipoproteins are specific proteins associated with lipoprotein particles, and they are either an integral part of the lipoprotein or are located peripherally. They can emulsify and transport lipids in the blood. They appear on the surface of the lipoproteins and a number are synthesized in the liver and intestine. They are apoB, (B-48 and B-100) apoA-I, apo A-II, apo A-IV, apo A-V, apoC-II, apoC-III and apoE. Apoproteins have important structural and metabolic functions. For example, apolipoproteins A are predominantly found in the high HDL and are important for reverse cholesterol transport (Zhao et al., 2012). Moreover, they are responsible for recognition of particles by receptors and act as coenzymes (enzyme activator) (Ginsberg, 2002, Gurr et al., 2002). **Table 1.2** illustrates sources and functions of apolipoproteins.

Table 1.2; Sources and functions of some lipoproteins (Jackson et al., 1976, Schaefer et al., 1978).

Apolipoprotein	Molecular wt.	Source	Function
apo A-I	28.300	Liver, Intestine	Structural component of HDL; activates LCAT
apo A-II	17.000	Liver, Intestine	May inhibit HL activity; inhibits AI/LCAT
apo A-IV	44.500	Intestine	Activates LCAT; possibly facilitates transfer of apos between HDL and CM
apo A-V	39.000	Intestine	Associated with lower TG levels; facilitates LPL
apoB-48	241.000	Intestine	Necessary for secretion of chylomicron from intestine, activates LPL uptake of remnants by the liver
apo B-100	55.000	Liver	Necessary for secretion of VLDL from liver; structural protein of VLDL, IDL and LDL; ligand for the LDL receptor
apo C-I	6.331	Liver	Activates LCAT; may inhibit hepatic uptake of CM and VLDL remnants; may inhibit CETP
apo C-II	8.837	Liver,	Activates LPL (essential cofactor)
apo C-III	8.764	Liver, intestine	Inhibits LPL and hepatic uptake of CM and VLDL remnants Ligand for LDL receptor, LDL receptor-related protein and proteoglycans
apo E	33.000	Liver (60-80%), and other tissues including adipose tissue	May inhibit HL activity; inhibits AI/LCAT

As each apolipoproteins has a specific function the proportions of them differ between lipoprotein particles (Schaefer et al., 1978) as shown in **Table 1.3**.

Table 1.3; Distribution of Apolipoproteins in different lipoprotein particles (Schaefer et al., 1978).

Apoprotein (%)	CM	VLDL	LDL	HDL
apo A-I	7.4	Trace		67
apo A-II	4.2	Trace		22
apo B-48	22.5			
apo B-100		36.9	98	Trace
apo C-I	15	3.3	Trace	1-3
apo C-II	15	6.7		3-5
apo C-III	36	39.9		+
apo E		13.0	Trace	

Two pathways are involved in lipoprotein metabolism; exogenous and endogenous. Exogenous pathway delivers cholesterol, TG and phospholipids from the small intestine to the liver and peripheral tissues, while the endogenous pathway transfers lipids from liver to peripheral tissue.

1.4.3 De-novo lipogenesis (DNL) pathway

In the DNL carbohydrates transformed into FA in the liver and adipose tissues (Hellerstein, 1999, Ameer et al., 2014). This pathway is activated in the feeding state

in which it is controlled by many hormonal factors such as insulin (Ameer et al., 2014). These factors include sterol response elements (SRE) binding protein isoform one (SREBP1-c) (Horton et al., 2002) and the liver X receptor- α (LXR α) (Liu et al., 2007b). It has been demonstrated that SREBP1-c upregulates all enzymes in the FA synthesis pathway and enzymes that regulate the availability of acetyl-CoA units in the pathway (Horton et al., 2002). Also, LXR α regulates lipogenesis via inducing the expression of SREBP-1c (Liu et al., 2007b). The Insulin increases the activity of LXR α , which hence induces SREBP-1c expression (Hellerstein, 1999, Ameer et al., 2014, Liu et al., 2007a). DNL contributes in FFA syntheses in endogenous pathway by 4% in fasted state and this rise to 8% postprandially. (Hellerstein, 1999, Ameer et al., 2014, Barrows and Parks, 2006).

1.4.4 Exogenous lipoprotein metabolism

The exogenous lipoprotein pathway starts in the intestine. Dietary triglycerides (approximately 100 grams per day) are hydrolysed to free fatty acids and monoacylglycerol by intestinal lipases and emulsified with bile acids, cholesterol, plant sterols, and fat soluble vitamins to form micelles. While the fatty acids in the intestine are overwhelmingly accounted for by dietary intake the cholesterol in the intestinal lumen is primarily derived from bile (approximately 800-1200 mg of cholesterol from bile vs. 300-500mg from diet) (Feingold and Grunfeld, 2015).

In the postprandial state, endothelial cells of the gut produce digested fat from food as CM, which contains varies components as seen in **Table 1.3**. These particles travel to plasma through lymph, and acquire the apoC-I, C-II, C-III and E from VLDL and HDL (Patsch, 1987). By acquiring apoC-II the action of LPL starts on CM hydrolysing TG to fatty acid and 2-monoacylglycerols. The hydrolysis continues until CM have lost 70-90% of their TG (Chen and Reaven, 1991). LPL cannot hydrolysis the TRL remnant particles and they are taken up by liver through specific receptors (Martins et al., 1997) (see **Figure 1.3**). It appears that apo B-48 containing particles are continuously secreted from the enterocyte and at times of excessive triglyceride availability, lipid

droplets fuse with nascent lipoprotein particles resulting in secretion of very large chylomicrons (Hayashi et al., 1990, Martins et al., 1994).

A balance between apoC-III, apoE which inhibit LPL and apoC-II which activates LPL is required for effective hydrolysis (Corrao et al., 1990). The CM remnant is generated by the lipolysis process. By definition, a TRL-remnant is an apo B-containing lipoprotein that has delivered some, or the major part, of its original triglyceride content to tissues by means of LPL-mediated lipolysis (Karpe, 1999). Certain other modifications also often take place, such as apoE enrichment, possibly apoC-III enrichment and apoC-II depletion. This new particle has more apoE and apoB and less apoA-I and apoCs with an abundant amount of CE. CE is transferred to other lipoproteins in exchange for TG, mediated by cholesteryl ester transfer protein (CETP) (Roche and Gibney, 2000). The CM-remnant is responsible for delivering cholesterol and TG to the liver (Redgrave, 2004). The liver uses lipid from this remnant to assemble VLDL (Jung et al., 1999). It has been shown that endogenous VLDL accumulate in the plasma postprandially due to delayed competitive lipolysis of apoB-100 TRL particles with CM for the sites of LPL action (Bjorkegren et al., 1996) (see Table 1.4).

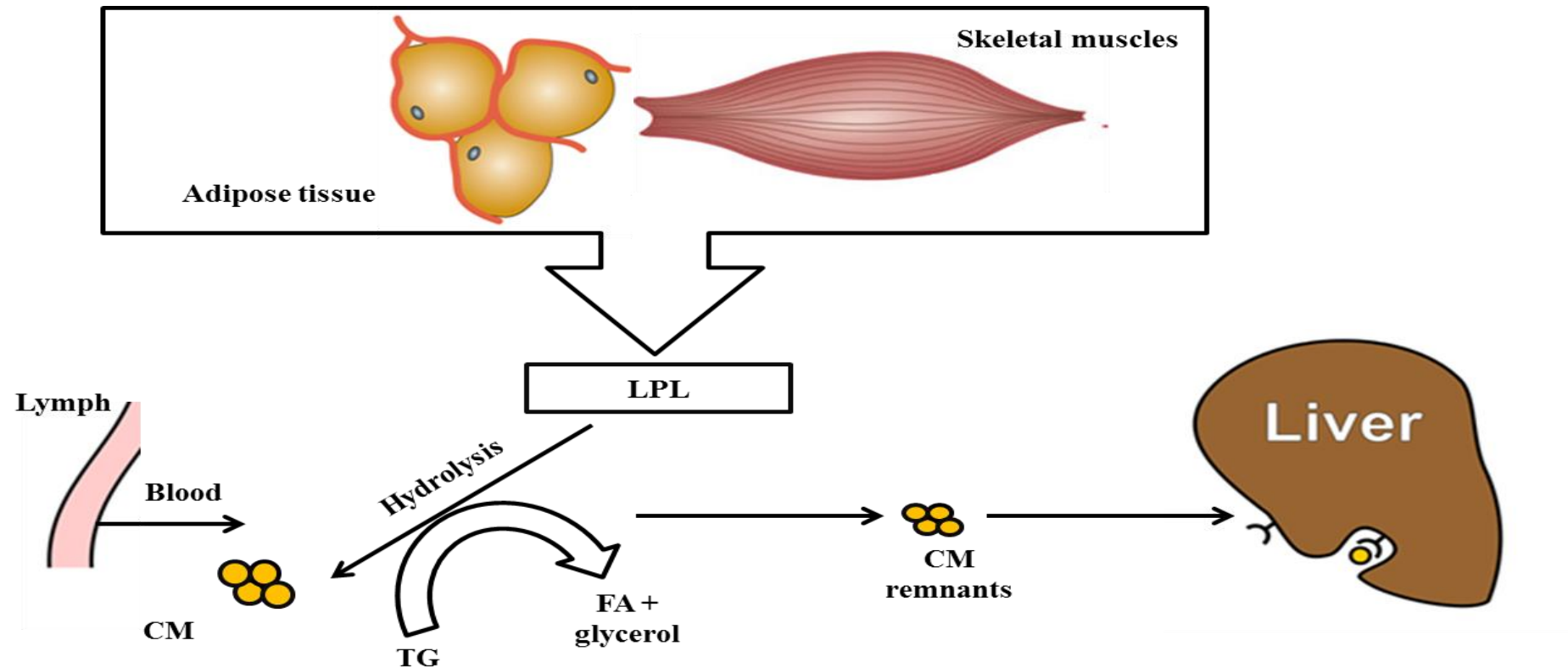


Figure 1.3; Chylomicron metabolism. Chylomicron metabolism begins when it emerges from the lymph carrying triglyceride into the circulation. Lipoprotein lipase from adipose tissue and skeletal muscles starts hydrolysing the triglyceride into fatty acids and glycerol. After it goes through the hydrolysis it loses most of its triglyceride, resulting in the formation of chylomicron remnants. These remnants enter to the liver through apoE receptors.

1.4.5 Endogenous lipoprotein metabolism

Metabolism in the post-absorptive state

Post-absorptive state refers to a state in which most of the previous meal has been fully absorbed *e.g.* after an overnight fast prior to any further food being consumed (Frayn 2009). The studies in subsequent chapters were implemented in the post absorptive state and therefore the following section will consider the metabolic processes involved in lipid metabolism during this state.

Lipoproteins synthesized by the liver transport endogenous TG and cholesterol. Lipoproteins circulate through the blood continuously until taken by peripheral cells or cleared by the liver. Factors that stimulate hepatic lipoprotein synthesis generally lead to elevated plasma cholesterol and TG levels.

VLDL metabolism parallels that of CM in many aspects. Like all lipoproteins, VLDL contains CE, phospholipids, free cholesterol and TG, and apolipoproteins as mentioned in **Table 1.3**. They are secreted continuously from liver and they are responsible for delivering TG to the peripheral tissues during fasting (**Figure 1.4**).

There are two subclasses of VLDL particles: large TG-rich VLDL₁ and smaller, more dense VLDL₂, which have more cholesterol and a lower ratio of apoCs and apoE to apoB (Packard and Shepherd, 1997, Packard et al., 1984). VLDL is continuously being excreted from the liver. A major proportion of the VLDL undergoes sequential delipidation to form LDL in the fasting state (Björkegren et al., 1997).

The secretion of the large VLDL particles is regulated by insulin-sensitive mechanisms which regulate the availability of triglyceride for VLDL production. FA are generated by lipolysis in adipose tissue through the action of HSL and are a major source for liver TG (Mifflin et al., 1990). Insulin stimulates the endothelial expression of LPL, the key enzyme for TRL metabolism, in a post-absorptive state (Semb and Olivecrona, 1986, Ong and Kern, 1989). Hepatic uptake of partially lipolysed VLDL or chylomicrons remnant particles may also contribute to the hepatocellular TG availability. Likewise, a lower rate of FA uptake by adipose and muscle tissues after LPL-mediated lipolysis of chylomicrons and VLDL promotes FFA uptake by the liver (Frayn et al., 1994). In addition, the liver has the capacity

of *de novo* synthesis of triglycerides, which is most evident in carbohydrate overfeeding. Therefore, the balance between oxidation of FFA in liver and muscle and the relative contribution of all of these TG/FFA flux rates, all regulated to a greater or lesser extent by insulin, determine the availability of hepatic TG for VLDL secretion (Malmström et al., 1997). Also, insulin seems to have a direct inhibitory effect on the hepatic secretion of large VLDL (Malmström et al., 1997). However, the secretion of smaller and less triglyceride rich VLDL, doesn't required any of the above stated mechanism. It needs cholesterol availability or a high cholesterol synthesis rate (Watts et al., 1995).

VLDL represents most of the pool of TRL in the post absorptive state. **Table 1.4** shows the variations of TRL concentrations during the day. It has been estimated that the endogenous TRL constitutes 96-97% of all TRL particles in the fasting state, and this figure was reduced to 91-96% in the postprandial state (Karpe et al., 1999, Sharrett et al., 1995).

Table 1.4; Percentage of TRL particles which are of endogenous origin (apo B-100) in the fasting and the postprandial state inhuman plasma (Karpe, 1999).

	0 h	3 h	6 h
Large TRL (S _f 60 - 400)	96 ± 3	91 ± 3	93 ± 4
Small TRL (S _f 20 - 60)	97 ± 2	95 ± 4	96 ± 3

It has been demonstrated that chylomicron and VLDL particles are competing for the same lipolytic pathway (Bjorkegren et al., 1996, Brunzell, 1973). After fat intake, an accumulation of endogenous TRL has been reported (Cohn et al., 1988a, Schneeman et al., 1993). This is might be due the delayed lipolysis of the apo B-100 TRL particles due to competition for the sites of LPL action by the chylomicrons (Brunzell, 1973). In rat plasma, it has been shown that the accumulation of endogenous TRL due the competition of the chylomicron-like triglyceride emulsion for the hydrolysis by LPL (Karpe and Hultin, 1995). In postprandial state, the rise of the number of TRL apoB-100 particles is actually far greater than that of apoB-48 containing lipoproteins (Havel, 1994). Conversely, 80% of the postprandial increase of triglycerides is accounted for by apoB-48

containing lipoproteins (Cohn et al., 1993). This apparent paradox is explained by the fact that the major triglyceride-carrier in the postprandial state is the chylomicron with each chylomicron particle carrying a very large number of triglyceride molecules. Therefore large quantities of triglycerides are transported by very few chylomicron particles (Karpe, 1999).

Within the circulation, LPL hydrolyses the TG in the core of VLDL. This action turns VLDL₁ into VLDL₂ which is then hydrolysed by the action of hepatic lipase (HL) and LPL to IDL, and HL then hydrolyses IDL to LDL (Nakajima et al., 2011b, Cohn et al., 1993). This delipidation process is slowed by the presence of chylomicrons resulting in a prolonged residence for VLDL in the postprandial state (Björkegren et al., 1997, Björkegren et al., 1996). This leads to more opportunities for cholesterol ester exchange between lipoprotein particles, and a cholesterol-enriched VLDL remnant is subsequently formed. This remnant lipoprotein is also enriched by apoE. Because of its increased cholesterol content, the remnant will probably not be further delipidated (Björkegren et al., 1997).

Cholesterol is an essential steroid involved in the formation of steroid hormones and bile salts and is also an important structural component of cell membranes (Hardman et al. 2003). LDL contains relatively high cholesterol content (around 40-50%) and transports the majority of cholesterol (Frayn, 2002). Binding of LDL to target tissue occurs through an interaction between the LDL receptor and apolipoprotein B-100 or E on the LDL particle. Uptake occurs through endocytosis, and the internalized LDL particles are hydrolysed within lysosomes, releasing lipids, mainly cholesterol (Packard and Shepherd, 1997). LDL is the supplier of body tissue with cholesterol; around 65% of total plasma cholesterol is carried in LDL (Wang and Briggs, 2004). Larger LDL particles exhibit the highest affinity (Packard and Shepherd, 1997). While 70% of the LDLRs are located on hepatic cells, 30% are located on the other cells of the body (Verges, 2005).

HDL metabolism

HDL particles are synthesized in the liver and the small intestine. They are a heterogeneous group of particles that differ in size, shape, density, cholesterol and phospholipid content, as well as in apolipoprotein such as, Apo A1, ApoE and Apo C-II (Miller, 1990). Apo A-1 activates LCAT, an enzyme associated to HDL (Von Eckardstein et al., 2001). They contain higher protein content, around 50 % of the particle total weight (Miller, 1990). The life cycle of HDL begins with apolipoprotein A-I being secreted by the liver (Miller, 1990). As ApoA-I bind circulating phospholipids and cholesterol, nascent discoid lipid-poor HDL particles are formed. These immature HDL particles trigger cholesterol efflux in sub-endothelial macrophages and fibroblasts and, via interactions with ATP-binding cassette transporter A1 (ABCA1) (Miller, 1990). This process is called Reverse Cholesterol Transport (RTC) which involves transport of cholesterol back to the liver for excretion or to other tissues that use cholesterol to synthesize hormones (Lewis and Rader, 2005). HDL particles assimilate cholesterol from cells into their cores, then the cholesterol is esterified by the action of LCAT. Finally, uptake of HDL cholesteryl esters by the liver, through the scavenger receptor B1 (SR-BI), hepatocytes and steroid-producing cells delivers cholesterol to sites where it can be metabolized or excreted in the bile (Lagrost and Gambert, 1991, Barter et al., 2003, Alan, 1993).

Mature HDL particles have a spherical shape. There are two main mature particles, HDL₂ and HDL₃ (Miller, 1990, Jaye et al., 1999). HDL particles acquire triglyceride via Cholesteryl Ester Transfer Protein (CETP) which promotes the transfer of cholesteryl esters and TG between HDL, LDL and VLDL (Lagrost and Gambert, 1991, Barter et al., 2003, Alan, 1993). CE is carried from HDL to VLDL and LDL particles, and TG is carried in the opposite direction from VLDL and LDL to HDL particles, resulting in CE depletion and TG enrichment of HDL (Lagrost and Gambert, 1991, Barter et al., 2003, Alan, 1993). The subsequent HDL particle enriched with triglycerides may regenerate small HDL particles through hydrolysis from hepatic lipase (Kontush and Chapman, 2006, Von Eckardstein et al., 2001, Lewis and Rader, 2005). It has been, demonstrated that the lower HDL concentration in the plasma the higher the risk of developing CHD (Gordon et al., 1989a) (see Table 1.5).

Table 1.5 Enzymes involved in lipoprotein metabolism (Gurr et al., 2002, Frayn, 2009).

Enzyme	Secretion place	Function
ABCA1	Within cells	Contributes to intracellular cholesterol transport to membrane
CETP	Liver	Mediates transfer of cholesteryl esters from HDL to VLDL
LPL	Endothelium	Hydrolyses triglycerides of chylomicrons and VLDL to release free fatty acids
LCAT	Liver	Esterifies free cholesterol for transport within HDL
HL	Endothelium	Hydrolyses triglycerides of VLDL ₂ , IDL, and LDL
HSL	Adipocyte cells	Hydrolyses triglyceride in adipocytes facilitating fatty acid release

ABCA1 = ATP-binding cassette transporter; CETP = cholesteryl ester transfer protein; LCAT = lecithin-cholesterol acyltransferase; LPL = lipoprotein lipase; HL= Hepatic Lipase; HSL= hormone-sensitive lipase.

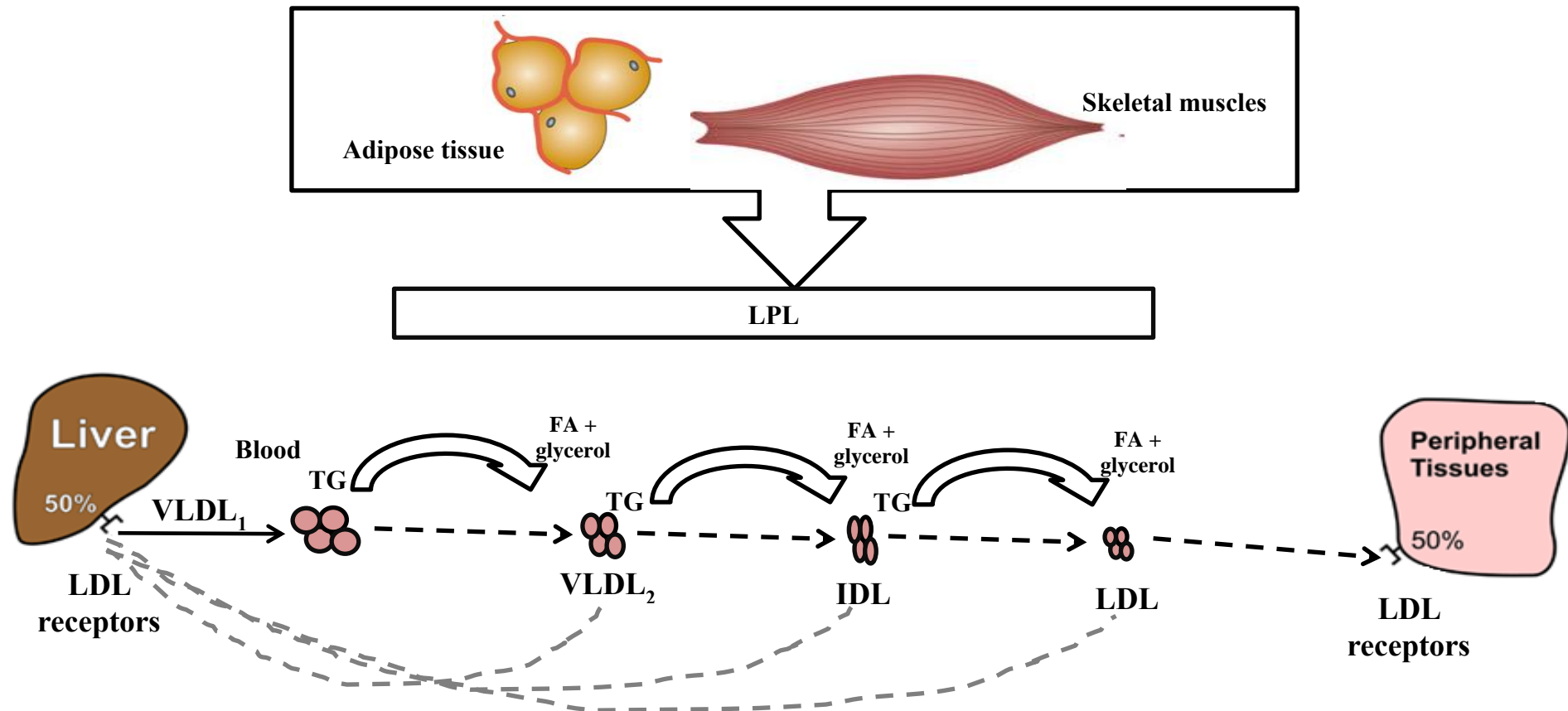


Figure 1.4. VLDL metabolism. VLDL is secreted from the liver with one apo B on the surface and triglyceride and cholesteryl ester in the core. Core triglyceride is hydrolyzed by lipoprotein lipase and becomes a remnant lipoprotein that is recognized by the liver in part, by apo E. The remnant lipoprotein is further processed to form LDL, which has a cholesterol-rich core and an intact apo B on its surface. The LDL particle can be removed by peripheral or hepatic LDL receptors. As the VLDL core is hydrolyzed, the unesterified cholesterol and phospholipid are transferred to HDL by phospholipid transfer protein to become the cholesteryl ester of HDL.

Function of Lipoprotein Lipase

Lipoprotein lipase (LPL) is a central enzyme in lipid metabolism. It is mainly produced by parenchymal cells of adipose tissue, heart and skeletal muscle (Merkel et al., 2002, Merkel et al., 1998, Olivecrona and Olivecrona, 1999). LPL is also produced in kidney (Ruge, 2004), lung (Hamosh and Hamosh, 1975), placenta (Lindegaard et al., 2005), brain (Vilaró, 1990) as well as in pancreatic islets (Cruz et al., 2001b) and in macrophages (Camps et al., 1990). LPL hydrolyses mainly TG, but also some phospholipids in TRL (Goldberg and Merkel, 2001, Olivecrona and Olivecrona, 1999). Moreover, LPL has the ability to bind simultaneously to lipoproteins and to specific cell surface receptors, such as the LDL receptor-related protein (LRP) (Beisiegel et al., 1991) and this allows it to carry out a non-catalytic, bridging function, which leads to the accumulation and cellular uptake of lipoproteins (Mead et al., 1999). These interactions lead to the increased accumulation and cellular uptake of lipoproteins. A number of studies have been provided evidence for this function of LPL. This bridging function has been reported in several tissue culture-based studies and *in vivo* (Mead and Ramji, 2002, Mead et al., 1999, Merkel et al., 1998). A variation of such bridging action involves cells rather than lipoproteins. For instance, LPL can act as a monocyte adhesion protein by forming a bridge between the monocyte surface HSPG and the arterial endothelial cells (Mamputu et al., 1997).

LPL activity is modulated by apoC-I, C-II and C-III (Jong et al., 1999). All three apolipoproteins are produced in the liver and they are physically associated with CM, VLDL IDL and HDL. Studies have provided support for a plasma TG-lowering effect of apoC-II via stimulation of LPL activity (Jong et al., 1999). It is widely known that apoC-II is an activator or co factor for LPL (LaRosa J. C., 1970). However, studies in mice have shown that the overexpression of the human apoC-II gene leads to marked hypertriglyceridemia via impaired plasma TG clearance (Hoogewerf et al., 1991), suggesting that at higher concentrations apoC-II may inhibit LPL (Jong et al., 1999). Several factors were shown to impact plasma apoC-II levels, including obesity diabetes and several hypolipidemic drugs (Kolset and Salmivirta, 1999). However, these factors are primarily pathological or pharmacological in nature, suggesting that

based on current knowledge apoC-II does not appear to be a major mediator of regulation of LPL activity in response to physiological stimulates, such as feeding/fasting, exercise and cold exposure. As opposed to C-II, C-I and C-III inhibit LPL-dependent plasma TG clearance, as shown using transgenic mice overexpressing human and mouse apoC-I or C-III (Nadanaka and Kitagawa, 2008, Pillarisetti et al., 1997, Wang et al., 2013) or mice lacking apoC-I (Stins et al., 1992)., It was proposed that apoC-I and C-III inhibit LPL activity by displacement of the enzyme from TG-rich particles (Chajek-Shaul et al., 1990). In addition, apoC-I and C-III may influence plasma lipoprotein metabolism via modulation of the activity of other enzymes involved in lipoprotein processing, as well as by altering the binding of apoC-containing lipoproteins to their receptors (Jong et al., 1999).

1.4.6 Plasma lipids as risk factors

Cholesterol and triglycerides, like many other essential components of the body, attract clinical attention when present in abnormal concentrations. Increased or decreased levels usually occur because of abnormalities in the synthesis, degradation, and transport of their associated lipoprotein particles. When hyperlipidaemia or hypolipidaemia are defined in terms of the class or classes of increased or decreased plasma lipoproteins, the names hyperlipoproteinaemia or hypolipoproteinaemia are preferentially employed (Stone, 2001, Havel, 1982). Increased concentration of plasma lipids is etiologically related mainly to genetic disorders, dietary factors (such as ingestion of excessive calories, trans and saturated fatty acids), or ingestion of some drugs, or it may occur as a secondary phenomenon in a large variety of diseases (Cox and Garcia-Palmieri, 1990, Stone, 2001). In any of these instances the elevation of the different plasma lipoproteins usually occurs in a number of combinations that have led to their classification into six different patterns or phenotypes (see Table 1.6).

Table 1.6. Lipoprotein Patterns Resulting from Elevation of Different Plasma Lipid Fractions (Cox and Garcia-Palmieri, 1990).

Lipoprotein pattern	Increased lipid fraction	Predominant lipoprotein
Type I	Triglycerides	Chylomicrons
Type 2a	Cholesterol	LDL
Type 2b	Cholesterol and triglycerides	LDL and VLDL
Type 3	Triglycerides and cholesterol	Remnants
Type 4	Triglycerides	VLDL
Type 5	Triglycerides and cholesterol	VLDL and chylomicrons

LDL as risk factor

Many studies have repeatedly shown that elevated levels of cholesterol play a key role in the development of atherosclerotic disease. In particular, LDL cholesterol has been strongly associated with CHD risk (Pyörälä et al., 1994, Expert Panel on Detection, 2001, Stamler et al., 1986, Castelli et al., 1992, Verschuren et al., 1995). LDL measurements are complicated by the fact that LDL is not a single-molecular species, but a multimolecular particle aggregate composed of protein and thousands of molecules of cholesterol and other lipids. Quantification can thus be accomplished in different ways, depending on which molecular constituent of LDL is measured. Because cholesterol is the most abundant lipid in LDL, and cholesterol assays have been available for many years, LDL concentrations in clinical practice are routinely expressed in terms of measured or estimated cholesterol content (LDL cholesterol [LDL-C]) (Friedewald et al., 1972). An elevated total serum low-density lipoprotein (LDL) cholesterol $\geq 4 \text{ mmol.l}^{-1}$, is considered as risk factor. An excess number of LDL

particles undergo oxidation and at hemodynamically vulnerable parts of the arterial vessels get trapped in the sub intimal space where they are taken up by monocytes that are transformed into macrophages (Pedersen, 2011). Ultimately these cells end up as foam cells in plaques and attract inflammatory molecules that further intensify the pathological process (Pedersen, 2011). Glycation is another type of atherogenic modification of LDL that may contribute to atherosclerosis (Soran and Durrington, 2011). Glycation is the result of bonding of a protein or lipid molecule with a sugar molecule, such as fructose or glucose, via a non-enzymatic process. Small, dense LDL is more susceptible to glycation than more buoyant LDL (Soran and Durrington, 2011, Ravandi et al., 2000).

A number of key studies provide the evidence base to understand the role of LDL in CHD. Total cholesterol was positively associated with IHD mortality in both middle and old age and at all blood pressure levels (Prospective Studies Collaboration, 1995, Prospective Studies Collaboration, 2007). Evidence that lowering LDL leads to reduced risk has come from landmark clinical trials. The Framingham study (considered the longest running study of cardiovascular disease in the world) which started in 1948 with an original cohort of 5,209 subjects aged 30-62 at baseline concluded that serum total cholesterol derived its atherogenic potential from its LDL component and showed also the importance of assessing the cardio-protective HDL fraction (Castelli et al., 1986). The total/HDL-cholesterol ratio was demonstrated to be the most efficient lipid profile for predicting coronary disease. LDL was shown to be correlated with hemostatic factors, suggesting that there would be additional benefits to lowering LDL (Castelli et al., 1986).

Important intervention trials helped proved the causal association between LDL cholesterol and CVD risk. The Scandinavian Simvastatin Survival Study (4S) further boosted the status of statins in preventive cardiology. A total of 4,444 patients with CHD and total plasma cholesterol 5.5-8.0 mmol.l⁻¹ on a lipid-lowering diet were randomly allocated on a double-blind basis to simvastatin 20-40 mg once daily or placebo for five years. There was an unequivocal 30% reduction in all-cause mortality ($P = 0.0003$), due to a 42% reduction in coronary deaths (Scandinavian Simvastatin

Survival Study Group, 1994). Even after 10 years of follow up, Simvastatin treatment for 5 years in a placebo-controlled trial, followed by open-label statin therapy, was associated with survival benefit over 10 years of follow-up compared with open-label statin therapy for the past 5 years only (Strandberg et al., 2004). The West of Scotland Coronary Prevention Study (WOSCOPS) was a landmark primary prevention trial that used pravastatin to reduce LDL cholesterol, (Packard and Shepherd, 1997). It showed again that statin use was associated with a reduction in CHD events. These studies and many other studies have shown the benefits of reduction in cholesterol on cardiovascular end points. The Heart Protection Study (HPS) was a landmark study in secondary prevention in which 20,536 people were divided into those given a statin and those on placebo. The active treated arm showed a substantial reduction in risk over the 5 years of the trial (Collins et al., 2002). For people with raised LDL who are at high CVD risk, drugs (statins) are considered essential. Dietary intervention leads to only a moderate lowering of cholesterol i.e. in the Whitehall Study in which 4469 patients completed the study, the patients who improved diet and lifestyle lowered cholesterol by only about 0.9 mmol.l^{-1} over 9 years compared with 2.7 mmol.l^{-1} for lipid lowering drug treatment (Bouillon et al., 2011). It has been observed that a reduction in plasma LDL-C concentrations by 1.0 mmol.l^{-1} reduces CVD mortality and non-fatal myocardial infarction by around 20-25% (Flather, 2010). It has been recommended to achieve a concentrations of 1.8 mmol.l^{-1} of LDL-C among high risk patients (Flather, 2010).

Small Dense LDL and CHD

Fisher *et al* proposed that small dense LDL may be more prevalent among CHD cases with premature atherosclerosis compared with controls (Fisher, 1983). The amount of TG transfer from TRL to LDL plays a crucial role in the formation of sdLDL. Thus, repeated episodes of exaggerated postprandial lipaemia can result in disturbances of the lipoprotein profile, characterized by elevated TG levels, increased hepatic VLDL-TG production, generation of sdLDL and a decrease in HDL concentrations. Small, dense LDL is thought to be particularly atherogenic because these particles have low affinity for LDL receptors and this will increase the residence time in the circulation and their opportunity for binding to the arterial wall (Chancharme et al., 1999).

Cross-sectional studies have reported that the odds of finding CHD among individuals with small, dense LDL particles was increased by 2-5 fold compared with individuals having larger, more buoyant LDL particles in Caucasian men (Campos et al., 1992, Tornvall et al., 1991, Coresh and Kwiterovich, 1996) and women (Austin et al., 1988, Coresh et al., 1993).

Other studies have explored further the atherogenicity of these LDL variants. Smaller and denser LDL particles are more susceptible to *in vitro* oxidation and taken up by macrophages and smooth muscle cells, leading to the development and progression of atherosclerosis (Carmena et al., 2004, Twickler et al., 2005, Tribble et al., 1994, de Graaf et al., 1991, de Graaf et al., 1993, Tribble et al., 1992), a mechanism that may contribute to the formation of foam cells *in vivo*. Small, dense LDL particles have also been shown to be degraded less rapidly than particles of intermediate density (Lamarche et al., 2008). This process has been attributed, among other factors, to the reduced binding affinity of small, dense LDL particles to the LDL receptor (Lamarche et al., 2008, Galeano et al., 1994). Small LDL particles also display an increased potential for interaction with proteoglycans of the arterial wall (Superko and Krauss, 1992, La Belle and Krauss, 1990), and appear to penetrate the endothelial barrier 1.7-fold more than large LDL particles (Rosenson, 2005). These processes could contribute to accelerate the formation of the atherosclerotic plaque and could explain, at least partly, the relationship between LDL particle size and density and the risk of CHD risk.

Role of HDL in the prevention of CHD

HDL is thought to protect against atherosclerosis in a number of ways including; removing cholesterol from foam cells, inhibiting the oxidation of LDL, and limiting the inflammatory processes that underlay atherosclerosis (Stender et al., 2005). The reduced plasma HDL cholesterol of $<0.9 \text{ mmol.l}^{-1}$ concentration characterizing hypertriglyceridaemic individuals with small dense LDL particles can be explained by an alteration of intravascular lipase activities. It has been shown that abdominal obesity was associated with a reduced plasma post-heparin lipoprotein lipase activity and with an increased hepatic lipase activity (Despres et al., 1990, Despres et al.,

1989) and these metabolic alterations may contribute to the hypertriglyceridemic-low HDL cholesterol dyslipidemia noted among subjects with small, dense LDL particles. The first study to show the negative relation between HDL and heart disease was the Framingham Heart Study in the 1980s (Brezina and Padmos, 1994). Supportively it has been demonstrated that independent negative association between HDL and ischaemic stroke mortality during a long-term (21-year) follow-up (Tanne et al., 1997). Another prospective study measured the survival in 535 elderly and they found that a significant negative association of serum HDL-C with mortality (Nikkla and Heikkinen, 1990). An estimated rise of 10 mg.dl⁻¹ of HDL-C is associated with a 2% lower risk of CHD for men and a 3% lower risk for women (Gordon et al., 1989b). A recent meta-analysis, including 302.430 subjects from 68 long-term prospective studies, supported the importance of HDL-C measurement in the risk assessment for CAD (Di Angelantonio E et al., 2009).

VLDL as a risk factor

Although LDL is the lipoprotein most commonly associated with atherosclerosis, other lipoproteins, such as chylomicrons and VLDL are also considered to be atherogenic (Khetarpal and Rader, 2015). A link between fasting plasma TG and CHD was first proposed in the 1950s (Gofman, 1953) and this was later confirmed in 1996 in a meta-analysis that showed that fasting TG levels are a weak risk factor for CVD (Austin et al., 1996, Austin et al., 1998). However, the association between fasting TG and CVD events is weakened or neglected in multivariate analysis which include HDL (Austin, 1990, Austin et al., 1996). This reported weak link between fasting TG levels and CHD is somewhat surprising, given that HDL-C concentrations which are mainly determined by the efficiency of TRL metabolism have a strong relation with CHD (Griffin, 1999). The perceived weakness of the association might be due the significant biological variation observed in fasting TG concentrations (Durrington, 1990, Tolfrey et al., 1999); this variations would lower its predictive power in multivariate analysis (Durrington, 1990). Moreover, fasting TG might not reflect the true state of the lipid's metabolism over a 24 hour period; postprandial plasma TG may be a more accurate and sensitive indicator of TRL metabolic efficiency. This has been supported by the observation that individuals with similar fasting plasma TG

have different levels of postprandial plasma TG (Patsch et al., 1983, Alcala-Diaz et al., 2014, Schrezenmeir et al., 1992, Corella and Ordovas, 2005).

Triglyceride concentrations in the postprandial period, are emerging as a significant independent risk factor for atherosclerosis, and may be a stronger predictor of cardiovascular disease and a contributor to insulin resistance compared with fasting TG levels (Langsted et al., 2011, Mora et al., 2008, Nordestgaard et al., 2007, Bansal et al., 2007, Schrezenmeir et al., 1997). Postprandial TG levels are well known to be a significant risk for CHD events (Mamputu et al., 2000, G Nordestgaard and J Freiberg, 2011, Patsch JR, 1992, Langsted et al., 2011). It has been reported also that postprandial TG has a stronger association to CHD than HDL-C concentrations (Patsch JR, 1992). Karpe (1999), found that a strong correlation between postprandial lipaemia and atherosclerosis. High concentrations of large VLDL (VLDL₁, S_f 60-400) are the major determinants of plasma TG levels and believed to initiate a chain of reactions that generate the atherogenic lipoprotein phenotype associated with insulin resistance, obesity, T2D and MetS (Taskinen, 2003, Ginsberg et al., 2005, Bloomgarden, 2007). However, the elevation of CM and CM-remnant concentrations might be as a result of the lipaemia that occurs due to impaired LPL function or any TRL and their remnants are the major lipoproteins which are increased in the postprandial hyperlipidaemia.

Chylomicrons as risk factors

Zilversmit was first to suggest that postprandial lipaemia as an important risk factor of CVD (Zilversmit, 1979). The increase in postprandial TRL from fasting levels contributed to approximately 80% of the increase of postprandial total TG from total fasting TG (Nakajima et al., 2011a). Individuals with hypertriglyceridemia tend to have a prolonged postprandial hypertriglyceridemia after a fat-meal tolerance test (Patsch JR, 1992). Exaggerated postprandial TG can occur at any age, as it has been observed in young women aged 22.5 years with existence of other CHD risk factors (Kolovou et al., 2011). Higher postprandial TG has been measured among diabetic adolescents (Umpaichitra et al., 2004), children with familial hypercholesterolemia (Reiber et al., 2003), and central obesity (Moreno et al., 2001) compared to control healthy participants. Evidence suggests that high postprandial concentration of TG can promote generation of potentially atherogenic TRL remnants (Karpe, 1999, Nakajima et al., 2009). It is also has been observed postprandial TG correlates more strongly with Remnant Lipoprotein (RLP) than fasting TG (Nakajima et al., 2009). It also induces the structural abnormalities in LDL and HDL particles (Karpe et al., 1993, Yang and Smith, 2007, Packard, 2003).

High concentrations of TRL in the postprandial state affect endothelial function and contribute to atherosclerotic plaque formation (Zilversmit, 1979, Groot 1991). An increased of CVD risk in men (32%) and women (76%) with elevated plasma concentrations of postprandial TG has been reported (Groot 1991, Patsch JR, 1992). Many other studies support Zilversmit's hypothesis, and show that patients with CHD have increased postprandial levels of TG and intestinally-derived TRL in response to a fat load challenge compared to healthy controls (Patsch JR, 1992, Groot 1991, Simons et al., 1987, Weintraub et al., 1996).

Plasma TG concentration fluctuates throughout the day in response to the ingestion of meals. Even if measured after a 10 to 12 hours overnight fast

(as is normal clinical practice), TG levels vary considerably more than LDL and HDL cholesterol levels (Nakajima et al., 2011b, Varbo et al., 2011).

Remnant lipoproteins and CHD risk

RLP, include chylomicron remnants and VLDL remnants which are subject to clearance by the liver (Mahley and Ji, 1999). They can enter the arterial wall, where they are preferentially retained and then taken up by macrophages. In macrophage-based studies, lipoprotein particles that increase sterol delivery or reduce sterol efflux or that promote an inflammatory response are considered atherogenic (Moore and Tabas, 2011). Compared with the nascent TG-rich lipoproteins, TRL remnants are depleted of TG, phospholipid, and apoC, but enriched in EC and apoE with a reduced size. Thus they are more likely to diffuse into the blood vessel wall, and retained by heparin sulphate proteoglycans (HSPG) within the arterial intima and can be taken up by macrophages (Nordestgaard and Nielsen, 1994, Tetali et al., 2010, Tomono et al., 1993). Therefore, they are more potentially atherogenic. Indeed, TRLP cholesterol level has recently considered as an independent risk factor for atherosclerosis and cardiovascular disease (Twickler et al., 2005, Nakajima et al., 2008). Postprandial RLP has also been shown to increase the expression of pro-inflammatory genes such as, interleukin-6, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and monocyte chemotactic protein-1 (Norata et al., 2007), induce apoptosis (Shin et al., 2004) and accentuate the inflammatory response of cultured endothelial cells to tumour necrosis factor- α (Ting et al., 2007). It has been suggested that the atherogenic potential of these lipoproteins is mainly due to their cholesterol content (Varbo et al., 2013). A non-fasting remnant cholesterol increase of 1 mmol.l⁻¹ is associated with a 2.8-fold causal risk for ischemic heart disease, independent of reduced HDL cholesterol (Varbo et al., 2013).

1.4.7 Obesity insulin resistance, diabetes and CVD risk

Prevalence and importance of obesity in CVD

Obesity and overweight are considered as one of the leading health issues and obesity is becoming a global epidemic (Kelly et al., 2008). The increase in the prevalence of obesity has occurred across every age, sex, and race (Hill and Melanson, 1999). Overweight and obesity are defined as abnormal or excessive fat accumulation of the adipose tissue that may impair physical and psychosocial health (Haslam and James, 2006, Naser et al., 2006). Overweight and obesity depend on the imbalance between energy intake and expenditure (Koopmans, 2003). To assess obesity Body Mass Index (BMI) is a widely used tool (see Table 1.7).

Table 1.7; Body Mass Index categories (WHO, 2012).

Category	BMI (kg.m ⁻²)
Under weight	16-18.5
Normal	18.5-24.99
Overweight	25.00 - 29.99
Moderately obese	30-35
Severely obese	>35

These BMI cut points in adults are the same for men and women, regardless of their age. For clinical and research purpose, obesity is classified into three categories: class I (30-34.9), class II (35-39.9), and class III (>40) (James et al., 2004). With the growth of extreme obesity, researchers and clinicians have further divided class III into super obesity (BMI 50-59) and super-super obesity (BMI > 60).

The current used BMI cut-off values are based on morbidity and mortality studies in Caucasian population (De Lorenzo et al., 2016). Several studies observed that some obese patients do not show expected metabolic abnormalities despite their substantial excess of body fat, demonstrating that while obesity increases the possibility of having complications, not every obese patient will develop them (Tchernof and Després, 2013). Although BMI is the accepted method to classify obesity and it can be used to predict and evaluate disease risk in epidemiological studies, it does not differentiate the composition of lean versus fat tissue and therefore may lead to erroneous interpretations (Sharma and Kushner, 2009). In addition to BMI and WC, there are other markers for excess body fat evaluation used for clinical practice, as the skinfold thickness and the waist-to-hip ratio (De Lorenzo et al., 2016).

Finally, direct measure of body mass fat, through magnetic resonance imaging (MRI), computed tomography (CT), dual-energy X-ray absorptiometry (DXA), bioimpedance analysis, and total body water, is gaining interest to assess the obese phenotype, but more studies are needed before either can be routinely recommended for office use.

Many large scale studies have shown a positive relationship between h CVD mortality and body mass index, a widely used measure of human obesity (McGee and Collaboration, 2005, Wilson et al., 2002, Stevens et al., 1998, Raben et al., 2003, Flint and Rimm, 2006, Calle et al., 2000, Larsson et al., 1984, Lapidus et al., 1984). Although cohort studies from Gothenburg, Sweden, in 1984 found that fat distribution was potentially stronger risk factor for morbidity and mortality (Lapidus et al., 1984, Larsson et al., 1984). Intra-abdominal fat has been associated with adverse clinical effects, characterized by hyperinsulinemia, dyslipidemia, glucose intolerance, and hypertension, increased risk of diseases such as type 2 diabetes, cardiovascular disease, and some cancers (Han et al., 1995, Bosello and Zamboni, 2000). Waist circumference for a given BMI was found to be a strong risk indicator of all-cause mortality in both men and women, and the combination of waist circumference and BMI may be very relevant in clinical practice (Bigaard et al., 2003). (see Table 1.8).

Table 1.8. Sex-specific cut-offs for waist circumference in different ethnic population (WHO, 2012).

Country / Ethnic group		Waist circumference
Europids In the USA, the ATP III values (102 cm male; 88 cm female) are likely to continue to be used for clinical purposes	Male	94 cm
	Female	80 cm
South Asians Based on a Chinese , Malay and Asian-Indian population	Male	90 cm
	Female	80 cm
Chinese	Male	90 cm
	Female	80 cm
Japanese	Male	90 cm
	Female	80 cm
Ethnic South and Central Americans		Same recommendations for South Asian until more specific data are available
Sub-Saharan Africans		Same recommendations for European data until more specific data are available
EMME (Arab) populations		Same recommendations for South Asian until more specific data are available

WHO statistics estimate the prevalence of overweight adults in 2014 was more than 1.9 billion and of these over 600 million were obese which is 13% of the world's adult population in 2014 (WHO, 2014b). Obesity is the sixth most important risk factor contributing to the overall burden of disease worldwide (Ezzati et al., 2002). In England in 2013, the percentage of adults aged 16 years and over measured as overweight or obese (BMI > 25) was 67 per cent in men and 57 per cent in women; the percentage measured as obese was 26% in men and 24% in women (Health and Social Care Information Centre, 2014). In Scotland in 2014, 69% of men and 61% of

women aged 16 years and over were overweight or obese; 26% of men and 29% of women were defined as obese (The Scottish Health Survey, 2014).

According to a recent health survey in the UK, the average prevalence of overweight and obesity (BMI > 25) is 66.1% in men and 57.5% in women, while the prevalence of obesity (BMI > 30) alone is 24.8% in men and 25.3% in women (NCD Risk Factor Collaboration, 2016). In the Middle East obesity has become a growing problem in the past two decades. The WHO indicates that the Gulf States have the highest rate of obesity and are in the list of top ten countries worldwide in term of obesity. In all WHO regions women were more likely to be obese than men. In the WHO regions for Africa, Eastern Mediterranean and South-East Asia, women had roughly double the obesity prevalence of men (WHO, 2014b, Alwan, 2011). Among different countries in the Middle East region there is significant heterogeneity in obesity prevalence (Sliem et al., 2012). Kuwait is the worst affected with a 42.8% obese population, placing Kuwait in the top 10 most obese countries in the world. Countries such as Saudi Arabia and Qatar are not far behind, with 35.2% and 33.1% obesity rates respectively (Arab Human Development Report, 2009). Obesity is assessed using body-mass index globally. Individuals are considered overweight with a BMI of 25 kg.m^{-2} or higher and obese with BMI of 30 kg.m^{-2} or more (WHO, 2014b). Obese individuals differ not only in respect to the excess fat mass but also in its regional distribution in different body sites. Indeed, central or visceral abdominal obesity is associated with substantially different metabolic profiles and cardiovascular risk factors than gluteal-femoral obesity. To assess these differences, it is useful to measure waist circumference (WC). Population studies have shown that people with larger WC have impaired health and increased cardiovascular risk compared with those with normal WC within the healthful, overweight, and class I obesity BMI categories. Abdominal fat is clinically defined as a WC of 102 cm or more in men and 88 cm or more in women (Sharma and Kushner, 2009).

Aetiology of obesity

Obesity is a multifaceted problem that can occur due to physiological, psychological and cultural factors. The aetiology of obesity is highly complex and includes genetic predisposition, physiologic, environmental, psychological, social, economic, and even political factors that interact in varying degrees to promote the development of obesity (Haslam, 2007, Haslam and James, 2006, Hill and Melanson, 1999). Obesity is most commonly caused by excess energy consumption (dietary intake) relative to energy expenditure (energy loss via metabolic and physical activity) (Jequier et al., 1987). Nutritional changes towards westernized diet, high in sugar and fats, and the sedentary lifestyle have led to increased obesity and CVD prevalence even in the developing countries (Sodjinou et al., 2008, Boutayeb, 2006, Popkin, 2002). Chronic positive shift of the energy equation resulting from increases in energy input, decreases in energy output, or both led to excess fat deposition in body (Bray, 1987). The imbalance in energy intake and expenditure does not need to be large to induce changes in body weight and an energy intake of just 5% higher than energy expenditure can result in 5 kg weight gain each year (Hill and Melanson, 1999). If repeated every year this has obvious implications for the development of obesity.

Energy balance is determined by macronutrient intake, energy expenditure and partitioning in nutrient storage (Bray, 1997). Thus, protein and carbohydrate intakes spontaneously elicit powerful autoregulatory adjustments in protein and carbohydrate oxidation, while the fat balance is less acutely regulated and more easily disrupted (Flatt, 1995, Schutz, 1995, Schrauwen et al., 1997). However, at the same time consumption of carbohydrates in excess of what is required may decrease internal fat oxidation which will contribute to a positive fat balance (Frayn, 2009). Although interventions on a person's nutrition can reduce BMI, it has been shown that efforts towards BMI reduction can be affected by a person's genetic profile (Arkadianos et al., 2007, Ordovas and Mooser, 2004). The synergy of genes and nutrition is studied within the new fields of nutrigenetics and nutrigenomics (Ordovas and Mooser, 2004). These new disciplines establish new strategies for CVD control which traditionally has been limited to nutrition interventions (e.g. fruits,

vegetables, fish) and supplementation, the latter being more popular in American population (Pearson et al., 2000). In addition, physical inactivity has dramatically increased in the past several decades and more time is spent on sedentary behaviours such as television watching, surfing the internet, and playing video games (Andersen et al., 1998).

Metabolic consequences of obesity

Obesity contributes to many metabolic abnormalities such as hypertension, IR, T2D and dyslipidaemia (Haffner, 2006) and organ damage such as fatty-inflammatory degeneration of the liver and peripheral vascular disease (Abate et al., 2001, Visscher and Seidell, 2001, Skilton et al., 2011). It is also a significant risk for the development of CHD and increased arterial stiffness (Zebekakis et al., 2005).

It is important to distinguish between android obesity and gynoid fat distribution, in which fat is allocated peripherally around the body (De Lorenzo et al., 2016). The type of obesity (android vs gynoid) and the amount of adipose tissue in the body could influence risk of developing CVD. Gynoid obesity, refer to preferential adipose tissue accumulation in the hips and thighs, with poor muscle-blood development, typically described as female obesity, a form much less associated with complications. Gynoid obesity is menaced only by direct mechanical complications of excessive adiposity: locomotor difficulty, abdominal pressure, limitation of respiratory motion, slowing of the venous and lymphatic circulation, cellulitis, lowering of energy, and reduction of the elasticity of the fat-infiltrated myocardium—complications which are all proportional to the degree of excess fat (Vague, 1956). Whereas, android obesity, refer to adipose tissue accumulated preferentially in the trunk/upper body area and pronounced muscle-blood development and suggested that this was a form of obesity closely associated with metabolic disturbances. It not only is associated with premature atherosclerosis and diabetes, but it is also the usual cause of diabetes in the adult in 80 to 90% of the cases. Gout and uric calculous disease generally appear in this form of obesity (Vague, 1956). Android obesity is associated with greater health risks such as increased TG and lower HDL concentrations in the plasma (Frayn 2002) and the progression of atherosclerosis (Kortelainen and Särkioja, 1999).

Accumulation of visceral fat contributes to a higher risk of hypertriglyceridaemia (Couillard et al., 1998), glucose intolerance and IR (Yang and Smith, 2007), all of which increase an individual's risk of developing CHD.

Interestingly the risks of diabetes, hypertension and dyslipidaemia increase from a BMI of about 21 kg.m^{-2} , thereby reducing life expectancy and greatly increasing socio economic burden (James et al., 2004). Evidence suggests that a 1 kg.m^{-2} increase in BMI increases the risk of developing new-onset T2D by 8.4%. The risk of impaired fasting glucose rises by 9.5%. An increase in waist circumference by 1 cm increases the risk of type 2 diabetes and impaired fasting glucose by 3.5% and 3.2% respectively (Bombelli et al., 2011). Moreover, it has been observed in the Asian Pacific study that, for each unit increase in BMI there is a 9% increase in ischaemic cardiac events and 7% of hypertensive death and strokes (Collaboration, 2004). Many mechanisms driven by obesity are thought to be responsible for this increased health risk and almost all are based on the unifying principle of generalized inflammation (Wellen and Hotamisligil, 2005, Wellen and Hotamisligil, 2003, Visscher and Seidell, 2001). Other mechanisms include impairment of glucose and insulin function.

Diabetes and insulin resistance

Insulin is a hormone produced by the β cells of the islets of Langerhans (Greenfield and Campbell, 2004, Kriketos et al., 2004, Dimitriadis et al., 2011). These cells are embedded in the exocrine portion of the pancreas (Greenfield and Campbell, 2004, Kriketos et al., 2004, Dimitriadis et al., 2011). Insulin is carefully coordinated with the release of glucagon from pancreatic α cells. The relative amounts of both hormones released by the pancreas are regulated so that the rate of hepatic glucose production is kept equal to the use of glucose by peripheral tissues (Greenfield and Campbell, 2004, Kriketos et al., 2004, Dimitriadis et al., 2011). The synthesis and release of insulin are decreased during the scarcity of dietary fuels and during stress. These latter effects are mediated by adrenaline which is a hormone secreted by the adrenal medulla in response to stress, trauma, or extreme exercise. On the other hand, insulin secretion and synthesis are increased by glucose, amino acids, FA and gut hormones (Greenfield and Campbell, 2004, Kriketos et al., 2004, Dimitriadis et

al., 2011). Insulin exerts important actions on metabolism as shown in **Table 1.9**. For instance, in the liver and muscle tissues, insulin increases glycogen synthesis, and glucose breakdown by increasing glycogenesis and glycolysis respectively. In muscle and adipose tissue, insulin increases glucose uptake by increasing the number of glucose transporters (GLUT-4) in the cell membrane. Also, in the liver, insulin decreases the production of glucose through the inhibition of glycogenolysis and gluconeogenesis (Greenfield and Campbell, 2004, Kriketos et al., 2004, Dimitriadis et al., 2011). Insulin also decreases TG degradation by inhibiting lipolysis in adipose tissue. This is accomplished by inhibition of hormone-sensitive lipase (HSL) activity which reduces the concentration of circulating FFA. Insulin also increases TG synthesis in adipocytes by increasing the rate of transport and metabolism of glucose into adipocytes, providing the substrate glycerol 3-phosphate for TG synthesis. Insulin also increases LPL activity in adipose tissue by increasing its synthesis, which increases TG hydrolysis releasing FAs needed for TG synthesis. Also, insulin in the liver promotes de novo lipogenesis, amino acid cellular uptake and protein synthesis (Greenfield and Campbell, 2004, Kriketos et al., 2004, Dimitriadis et al., 2011).

Table 1.9; Insulin's effects on metabolism.

Inhibit	Stimulates
Gluconeogenesis	Glucose uptake
Glycogenolysis	Glycolysis
Lipolysis	Glycogenesis
Ketogenesis	Protein synthesis

An altered cellular biological response to insulin action is known as IR, and it is associated with T2D, obesity and metabolic syndrome (Greenfield and Campbell, 2004, Kriketos et al., 2004). Therefore, IR is a strong sign of the risk of developing

T2D especially in obese individuals who are present with central or visceral obesity (Greenfield and Campbell, 2004, Kriketos et al., 2004).

Insulin resistance, defined as inhibition of insulin stimulation of several metabolic pathways including glucose transport, glycogen synthesis and anti-lipolysis, is of considerable clinical relevance because it is pathophysiologically linked to several serious medical problems including type 2 diabetes (Boden, 2011). Type two diabetes is a metabolic disorder of the haemostasis of body fuel characterized by a decreased rate of insulin-mediated glucose uptake due to peripheral insulin resistance (Kasuga, 2006). The pathology of diabetes is a complex process and it is accompanied by a variety of metabolic abnormalities attributed to IR (Atkinson and Maclaren, 1994, Baekkeskov et al., 1982, Catalano et al., 2014). Insulin sensitivity fluctuation occurs across through life cycle for example, during puberty, in pregnancy, and during the aging process (Karpe et al., 2011). Generalized insulin resistance occurs primarily as a result of obesity. Insulin resistance is associated with many serious medical conditions, such as type 2 diabetes, hypertension, atherosclerosis, and metabolic syndrome (Boden, 2011, Reaven, 1988).

Adipose tissue affects metabolism by secreting hormones, glycerol, and other substances including leptin, cytokines, adiponectin, and pro-inflammatory substances, and by releasing NEFA. In obese individuals, the secretion of these substances will be increased (Karpe et al., 2011). Insulin sensitivity is inversely proportional to adiposity (Yki-Järvinen and Koivisto, 1983). Body fat distribution also has an influence on insulin sensitivity. Insulin resistance is associated with body mass index at any degree of weight gain. Insulin sensitivity also differs completely in lean individuals because of differences in body fat distribution. Individuals whose fat distribution is more peripheral have more insulin sensitivity than do individuals whose fat distribution is more central (Karpe et al., 2011). Abdominal fat is considered more lipolytic than subcutaneous fat, and it also does not respond easily to the antilipolytic action of insulin, which makes intra-abdominal fat more important in causing insulin resistance, and thus diabetes (Roden et al., 1996, Fain et al., 2004).

NEFA levels are strong predictors of muscle insulin resistance (Kelley et al., 2001). Increased release of NEFAs is observed in T2D and in obesity, and it is associated with insulin resistance in both conditions (Jelic et al., 2007). Muscle fat content is increased in obesity and more so in type 2 diabetes (Kelley et al., 2001). Shortly after an acute increase of plasma NEFA levels in humans, insulin resistance starts to develop. Conversely, when the level of plasma NEFA decreases, as in the case with antilipolytic agent use, peripheral insulin uptake and glucose monitoring will be improved (Roden et al., 1996). Circulating concentrations of plasma NEFA are determined to a large extent by the release by lipolysis of adipocyte triglyceride stores by adipose lipase (AL) (Lass et al., 2011) and HSL. Hormone-sensitive lipase stimulated release of FFA from TG stores in adipose tissue, is tightly controlled by hormones that are regulated by the metabolic status. During conditions such as fasting, when blood glucose is low or when energy demands are increased, glucagon, glucocorticoids and catecholamines lead to activation of HSL to promote hydrolysis of stored triglycerides to FFA (Stanley et al., 2005). By contrast, in the fed state insulin inactivates HSL and inhibits lipolysis (Stanley et al., 2005). In vivo, the majority of FFA that are delivered to tissues arise from hydrolysis of a triglycerides, which are transported in plasma in chylomicrons or VLDL particles and the remainder exist in the non-esterified form bound to albumin. Plasma FFAs can increase in healthy individuals due to adrenergic stimulation brought on by exercise, stress, fasting, ischemia, or diabetes. The release of FFA from chylomicrons or VLDL by lipoprotein lipase in these situations also increases plasma FFA (Stanley et al., 2005). When tissues up take FA, they have three major fates in the cell. They can be esterified into TG, diglycerides, or phospholipids; converted to sphingolipids; or oxidized for energy (Chavez and Summers, 2010). FFAs are transported to cardiomyocytes by either passive diffusion or transport proteins (Stanley et al., 2005). Since the majority of FFA that enter the heart are to provide 70-90% of the energy (Lopaschuk et al., 1994), they must enter the mitochondrial matrix for β -oxidation. FFA are transported across the outer and inner mitochondrial membrane by carnitine palmitoyl transferase 1 (CPT1), which is the rate-limiting step of fatty acid oxidation, and CPT2. The acetyl-CoA enters the tricarboxylic acid cycle, yielding NADH and FADH₂, which enter the electron transport chain to produce ATP (Stanley

et al., 2005). Persistent exposure of tissues to increased concentrations of fatty acids, and associated changes in the metabolic fate of fatty acids are an important cause of insulin resistance.

The resistance to insulin action in diabetes is due to reduced insulin action on target tissues such as skeletal muscle and adipose tissues (Atkinson and Maclaren, 1994, Baekkeskov et al., 1982). The normal effect of insulin on carbohydrate metabolism is reducing glucose levels on the plasma, on lipids it favours its synthesis (lipogenesis) and decreases its breakdown by favouring cholesterol biosynthesis by using glucose as substrate and also favours protein synthesis by decreasing protein catabolism. The dysregulation of insulin action on carbohydrate, protein and lipid metabolism causes the clinical condition 'hyperglycaemia' (Falciglia, 2007, Triplitt et al., 2015). Still it is highly debatable about the events that are causing T2D. It is associated with adverse changes in cardiovascular risk factors such as high TG levels, low levels of HDL-C and raised blood pressure. It usually results from impairment of β cells to secrete adequate insulin in response to overeating, inactivity, obesity and IR (Weyer et al., 1999, Schrauwen-Hinderling et al., 2011). It has been observed that patients with diabetes are more likely to have high plasma TG concentration (Shen, 2007). In the San Antonio Heart Study patients with insulin resistance were at high risk for CVD development and presented with elevated TG, blood pressure and lower HDL compared with healthy non-diabetic individuals (Haffner et al., 2000). IR may be the underlying cause of impaired glucose tolerance (Weyer et al., 2001, Garvey and Hermayer, 1998). The exact mechanisms that cause IR are not fully understood and both environmental and genetic factors are involved. IR has been linked with one or more genes with varying frequencies among different ethnic groups (Mercado et al., 2002, Stern and Mitchell, 1999).

Diabetes and CVD risk

According to IDF (International Diabetes Federation, 2014), there are currently 382 million people with diabetes, which is projected to rise to 592 million by 2035. Over three-quarters of these cases are in low middle-income countries (International Diabetes Federation, 2014, Shaw et al., 2010, Whiting et al., 2011). Diabetes caused

around 4.9 million deaths in 2014 (International Diabetes Federation, 2014, Shaw et al., 2010, Whiting et al., 2011). Diabetes and IR are prime risk factors for CVD. Diabetes leads to metabolic, structural and functional changes in the heart and vasculature leading to diabetic cardiomyopathy, coronary artery disease and myocardial ischemia, and ultimately heart failure (Gray and Kim, 2011, Donnelly et al., 2000). Along with complications like retinopathy and nephropathy, peripheral vascular disease (PVD), it is also one of the leading risk factors for stroke and coronary artery disease (CAD). As people progress from impaired glucose tolerance (IGT) to diabetes mellitus, their risk of coronary heart disease (CHD) and stroke increases 2 to 3 fold (Wilson et al., 1991, Stamler et al., 1993b, Zimmet et al., 2001), while the risk of peripheral vascular disease increases by four-fold (Haffner et al., 1998). The risk of all cause and CVD mortality is almost double in people with glucose levels $\geq 11.1 \text{ mmol.l}^{-1}$ in the fasted state, measured in response to an oral glucose load (Shaw et al., 2010). Diabetes also affects the heart muscle causing heart failure (Dokken, 2008, Muhlestein et al., 2003, Thrainsdottir et al., 2005). A possible explanation is the impact of insulin resistance to induce impaired vascular function, which leads to impaired nitric oxide mediated vasorelaxation, which may contribute to hypertension and to increased risk of atherosclerosis (Zhang et al., 2012, Symons et al., 2009, Muniyappa et al., 2008). Moreover, genetic manipulation of insulin action in the vasculature will increase atherosclerosis (Rask-Madsen et al., 2012, Rask-Madsen et al., 2010, Qiang et al., 2012). Insulin resistance via multiple mechanisms may contribute to macrophage accumulation in the vessel wall to increase atherosclerosis and instability of vulnerable plaques (Bornfeldt and Tabas, 2011, Aurigemma et al., 2013). Lastly, insulin resistance has been shown in many human and animal studies to increase the extent of myocardial injury in the context of myocardial ischemia, which may contribute to the increased risk of heart failure in affected individuals (Aurigemma et al., 2013, Abel et al., 2008).

Causes of diabetic dyslipidemia

Diabetic dyslipidaemia is caused by several factors, including hyperglycaemia, insulin resistance, hyperinsulinaemia, visceral adiposity, hepatic steatosis, and dysregulated

fatty acid metabolism (Taskinen, 2003, Chan and Watts, 2011). Insulin is involved in the regulation of lipid metabolism particularly in the release of FFA, as patients with IR or diabetes have higher TRL level and abnormalities in their lipid profile (Taskinen, 2003). Any impairment of insulin sensitivity appears in the liver, skeletal muscles and adipose tissue (Turner and Clapham, 1998) and causes impairment in glucose uptake and storage (Turner and Clapham, 1998). Insulin resistance leads to an increase in the burden of fatty acid which builds up in the liver as triglyceride. The liver has three options for handling excess TG: store them, enhance β -oxidation in mitochondria (Gavrilova et al., 2000, Turner and Clapham, 1998, Meena et al., 2014, Shen, 2007, Björntorp, 1991, Wood, 2006) or stimulate the synthesis VLDL resulting in hypertriglyceridemia (Steinberger et al., 2001, Björntorp, 1991, Reaven, 1988, Reaven and Chen, 1996, Wood, 2006).

In patients with T2D have high concentrations of both fasted and postprandial TG and apoB-48 (Lewis et al., 1991, Meng et al., 1983, Schaefer et al., 2002, Taniguchi et al., 2000, Curtin et al., 1996). Elevated concentrations of postprandial CM and CMRs in insulin resistance have been mainly attributed to impaired TRL clearance from the circulation (Shojaee-Moradie et al., 2013). In patients with T2D, increased plasma apoB-48 is related to reduce the rate of CMRs catabolism (Dane-Stewart et al., 2003, Hogue et al., 2007). Therefore, clearance of CMRs could be impaired as a result of increased hepatic-VLDL secretion in insulin resistance (Lewis, 1995). That is because increased availability of TG and cholesterol in T2D increases VLDL-hepatic synthesis which might affect the LPL activity in clearing CMR (Lewis, 1995).

The overproduction of VLDL is a key mechanism leading to the development of dyslipidaemia in the metabolic syndrome and a frequent co-morbidity of peripheral IR and type 2 diabetes mellitus (Bamba and Rader, 2007). This resulting overproduction of VLDL₁ causes an increase in small density LDL and decreases HDL transport to liver (Duell et al., 1991, Zimmet et al., 2001).

Insulin resistance also contributes to elevated TG levels in plasma by reducing the activity of LPL which inhibit the clearance of both VLDL and TRL (Mead et al., 2002).

The slow removal of CM and CMRs by reduced LPL activity has been addressed in insulin resistance (Kobayashi et al., 2007) due to the diminished regulation of LPL by insulin (Patsch, 1998). Moreover, diabetic patients in general and diabetic women in particular have slow clearance of CM which expose the arterial wall to high concentrations of accumulated VLDL and CM (Goldberg and Merkel, 2001, De Man et al., 1996, Howard, 1999).

Formation of small dense LDL in diabetics

CETP promotes exchange of HDL cholesteryl ester for triglycerides of TRL. In the presence of high concentrations of chylomicrons and VLDLs, HDL cholesteryl esters are preferentially transferred by CETP to larger VLDL particles that become cholesterol rich and thus, potentially more atherogenic (Guérin et al., 2001). Transfers of cholesteryl esters to TRLs, particularly chylomicrons, are enhanced in the postprandial state (Guerin et al., 2002, Contacos et al., 1998). The rate of cholesteryl ester transfer to TRLs and LDLs and the mass of CETP are increased in patients with a range of atherogenic dyslipidaemias (McPherson et al., 1991). Thus, reducing VLDL₁ concentration is likely to induce clinically important changes to the atherosclerotic risk profile.

Other studies show that insulin directly increased degradation of newly synthesized apoB (Sparks and Sparks, 1990). Therefore, insulin deficiency or hepatic insulin resistance may increase the secretion of apoB. Insulin may modulate the production of a number of other proteins that affect circulating levels of lipoproteins. These include apoC-III (Chen et al., 1994), a small apoprotein that may increase VLDL by preventing the actions of LPL and inhibiting lipoprotein uptake via the LDL receptor-related protein (LRP). This leads to decrease in the clearance of postprandial remnant lipoproteins. LDL particles in diabetic patients can also become glycated, in a process similar to the glycation of the haemoglobin. Glycation of LDL lengthens its half-life (Napoli et al., 1997) and therefore increases the ability of the LDL to promote atherogenesis (Bucala et al., 1993).

HDL acquires cholesterol and PL by the actions of phospholipid transfer protein (PLTP) or by the efflux of cellular FC by the esterification of this cholesterol by the enzyme

lecithin cholesterol acyl transferase (LCAT) (Hayek et al., 1993, Jiang et al., 1999, Young and Fielding, 1999). This metabolism is often defective in diabetes, reducing the production of HDL-C from this source (Taskinen, 1987). Also, high concentrations of TRL, stimulate CETP exchange of VLDL triglyceride for cholesteryl ester in the core of LDL and HDL. This triglyceride can then be converted to free fatty acids by the actions of plasma lipases, primarily hepatic lipase. The net effect is a decrease in size and an increase in density of both LDL and HDL (Chahil and Ginsberg, 2006). (see **Figure 1.5**).

Dyslipidaemia is only one mechanism by which diabetes promotes atherosclerosis. IR is also believed to be a key factor mediating the progression of endothelial dysfunction (Lteif et al., 2005). Moreover, diabetes might also influence CVD by leukocyte adhesion, thrombogenesis and inflammation (Celermajer, 1997, Pickup and Mattock, 2003). The latter also has a paracrine suppressive effect on the secretion of adiponectin, a powerful insulin sensitizer which is secreted less as the adipocyte mass expands (Ryo et al., 2004, Kojima et al., 2005, Friedman and Halaas, 1998). The fat accumulation into islet cells might cause a reduction in the capacity of islets ultimately causing glucose intolerance and premature T2D (Ouchi et al., 1999, Kojima et al., 2005).

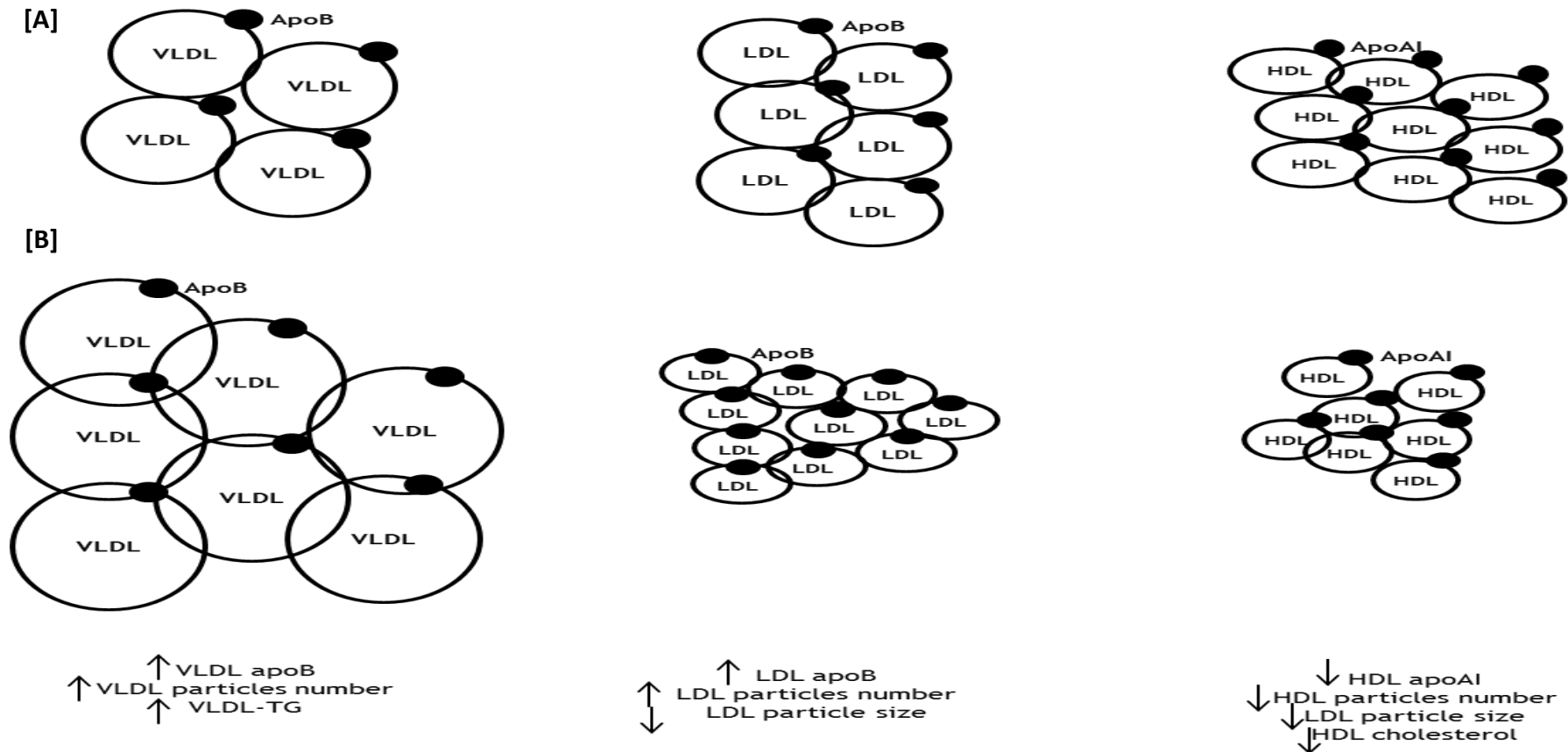


Figure 1.5. Dyslipidaemia of insulin resistance. Panel [A], shows the normal lipedmic response, Panel [B] shows insulin resistance/diabetes dyslipidaemia. Hypertriglyceridaemia reflects accumulation in plasma of TRL, the pivotal defect in lipoprotein metabolism. Over secretion of VLDL and chylomicrons by the liver and intestine, coupled with decreased catabolism, increases the plasma pool of TRLs, including remnant lipoproteins; increased hetero exchange of neutral lipids between TRL and LDL and HDLs via CETP results in remodelling of LDL and HDL to form correspondingly smaller, denser particles. LPL activity is decreased in skeletal muscle and adipose tissue owing to the inhibitory effects of insulin resistance and apoC-III.

1.5 Intervention to modify CVD through behavioural changes

Most patients often undergo pharmacological treatments for lipoprotein disorders, obesity and IR. Long-term pharmacological therapies are often expensive, and probably undesirable to large sections of the population, whereas life style, diet and exercise can be an alternative solutions that are more acceptable and carry less risk of side effects.

1.5.1 Controlling plasma lipids by exercise

Moderate exercise is one potential non-pharmacological treatment to reduce elevated postprandial TG concentrations. In the last decade studies have shown that exercise can lower TG concentrations in both fasting and postprandial state by 20-25%, mostly the VLDL₁ particles among highly risk CVD population (Gill et al., 2004a) such as centrally obese middle-aged men (Gill et al., 2004a) and postmenopausal women (Gill and Hardman, 2000). This effect is seen following a single exercise session and is not due to weight loss (Gill et al., 2004b, Gill et al., 2004a). The TG-lowering effect cannot be replicated by a dietary-induced energy deficit of the same magnitude (Gill and Hardman, 2000) although it contributes as replacing the energy expended by exercise attenuates the TG-lowering effect (Burton et al., 2007). However, exercise induces lower postprandial TG concentrations when food is consumed ad libitum (Farah and Gill, 2012).

Various studies indicated that exercise reduces postprandial lipaemia in various age groups and usually the effect of exercise appears after some hours of after exercise (Peterson et al., 1990).

Borghouts et al., indicated that insulin sensitivity is improved by exercise training (Borghouts and Keizer, 2000). Mestek et al., (2009) reported that, in men with MetS, postprandial lipaemia tends to be lower after continuous aerobic exercise with 500 kcal of energy expenditure before a meal (Mestek, 2009). Ziogas et al., (1997) demonstrated that endurance exercise individuals exhibit a significantly lower postprandial hyperinsulinaemia response to a fat-rich meal compared with sedentary individuals (Ziogas et al., 1997). Ten hypertriglyceridaemic men with

insulin resistance were studied by Zhang *et al.*, (2007), and they suggested that in individuals with metabolic syndrome, exercising at moderate intensity for 45 min effectively resulted in a reduction of postprandial hypertriglyceridemia, while exercise for 30 min is sufficient to improve insulin action (Zhang *et al.*, 2007). Similarly, Schrauwen-Hinderling *et al.*, (2011) studied the effect of exercise on 11 diabetic patients for 12 weeks of progressive endurance/strength training and found that although the cardiac lipid content remained unchanged, the aerobic capacity and insulin sensitivity were increased (Schrauwen-Hinderling *et al.*, 2011). Lee *et al.*, (2011), investigated the effect of exercise on diabetic mice and implied that adiponectin (APN) suppresses inflammation and oxidative stress in the aorta (Lee *et al.*, 2011). Supervised aerobic training exercise for 16 week in dyslipidemic patients without MetS was associated with weight loss, increased APN and a decline in LDL, IDL and VLDL-cholesterol (Yoshida *et al.*, 2010). Endurance exercise training is known to generally increase HDL cholesterol concentrations and to decrease plasma triglyceride and LDL cholesterol levels (Després and Lamarche, 1993, Després and Lamarche, 1994).

Studies show that trained individuals have lower postprandial lipaemia than untrained (Ziogas *et al.*, 1997) and 50% lower TG concentration after exercise (Merrill *et al.*, 1989). The period of endurance has been studied in different groups of individuals: overweight men and women with dyslipidaemia (Kraus *et al.*, 2002) and older men and women (Halverstadt *et al.*, 2007), and these indicate that the period of training reduces either fasting and/or postprandial TG. However, the effect on TG is due to short term metabolic response as shown by Herd *et al.*, (2000) who found no difference in postprandial TG concentrations between endurance trained and untrained young adults. The basic role of exercise depends on how much energy is expended, according to a study conducted in 2008 by Burton *et al.*, on 13 overweight/ obese men who enrolled for a three arms namely control, energy defect and energy replacement (Burton *et al.*, 2007). The authors found that the greater reduction in postprandial TG was with energy defect by 14%, compared to with 10% observed control and energy replacement arms respectively.

Comparing regular exercise with a single bout of exercise

Regular exercise is beneficial for health by maintaining the healthy weight and it reduces TG concentration in the plasma (Jakicic, 2002). It also helps to reduce the acute changes in TG metabolism (Peterson et al., 1990). It has been found that there is no difference on level of TG on plasma between 30 min of moderate cycling (0.87 MJ per 30 min) accumulated in short bouts and one continuous 30 min bout of cycling in obese men (Miyashita, 2008). Exercising at moderate activity over the course of 6 months, was shown to significantly increase HDL-C in young adult women (Duncan et al., 1991). In a population of men and women ranging from 50 to 65 years of age, however, an increased frequency of exercise resulted in the highest HDL-C levels (Kraus et al., 2002). Over the course of only 3 weeks of exercise, although HDL-C levels did not increase, HDL preferentially converted to an anti-inflammatory state (Roberts et al., 2006). A beneficial effect on LDL particle size and density has been observed in long -distance runners (Williams et al., 1986).

On the other hand, many studies show that a single session of exercise is more effective on TG reduction than continuous exercise. A session of aerobic exercise tends to reduce postprandial hyperinsulinaemia in healthy adult (Tsetsonis and Hardman, 1996, Zhang et al., 2007, Graham, 2004). Moreover, Mestek *et al.*, (2009), stated that accumulating moderate-intensity exercise does not appear to effectively modulate postprandial lipaemia in men with MetS (Mestek, 2009). Similarly, different kinds of exercise either in its intensity or duration have the same influence in decreasing plasma TG (Tsetsonis and Hardman, 1996). The greatest effect of exercise appears 12-18 h after exercise (Peterson et al., 1990). Similarly exercise is considered to have short lived effect on insulin because insulin sensitivity can decline significantly after as little as 38 h after the final exercise training (Mikines et al., 1989). One single 90 minute session of moderate intensity exercise completely reversed FFA induced insulin resistance in healthy volunteers one day later (Schenk and Horowitz, 2007).

Energy Expenditure and Energy Deficit

Accumulating evidence suggests that the TG lowering effect of exercise depends on the energy expended during the exercise sessions (Gill et al., 2003). It is widely accepted that the higher the energy expenditure, the larger amount of TG reduction (Petitt and Cureton, 2003). Maraki *et al.*, (2009) studied the influence of low energy expenditure exercise (30% of maximal oxygen uptake (VO_{2max}) along with mild energy intake restriction by 2.44 MJ on fasting and postprandial TG in young women (Maraki et al., 2009). They demonstrated that, fasting plasma TG, TG in TRL and serum insulin concentrations reduced by 18, 34 and 30% respectively after intervention compared with the control trial. Postprandial concentrations of plasma TG and TRL-TG also reduced significantly but there was no difference in serum insulin concentrations. Although energy intake restriction can reduce fasting TG, postprandial lipaemia and insulin concentrations, exercise was more effective in reducing fasting and postprandial TG and insulin in postmenopausal women (Gill and Hardman, 2000). Another study by Burton et al., indicated an approximately doubling of the reduction in postprandial lipaemia and rise in fat oxidation through exercise alone when compared to exercise plus energy replacement (Burton et al., 2007).

Another way to manipulate the energy expenditure is to increase the duration of exercise. For instance significant reductions of both postprandial TG and postprandial insulin response was showed in premenopausal women who exercised for 2 h vs. 1 h at 50% VO_{2max} another (Gill et al., 2002). They found that the reduction of postprandial TG concentrations was more after 90 min exercise at 60 % of VO_{2max} compared to the same period at 30% VO_{2max} (Tsetsonis and Hardman, 1996). Magkos *et al.*, in different study found that a 2 h cycling at 60% decreased VLDL-TG -but not VLDL-apoB at the same intensity (Magkos et al., 2007, Magkos et al., 2006). As the exercise intensity and duration reduce postprandial TG, it also increases insulin sensitivity. It has been suggested that the effect of exercise on insulin sensitivity can last for 15 days on middle age overweight men which depends on intensity and weekly amount of exercise during each session (Breckenridge et al., 1982). Babraj and his team reported that low volume, high intensity exercise is effective in reducing plasma glucose and insulin in sedentary young adults (Babraj et al., 2009).

Possible Mechanisms for the effect of exercise on TRL

The TG lowering effect of exercise does not appear to be due to a reduction in the rate of appearance of CM in the circulation (Gill et al., 2006, Gill et al., 2001a). There is also no impact of exercise on CM number (James et al., 2007).

Evidence supports the hypothesis that endurance trained individuals have high clearance rates of CM-like lipid emulsions compared with untrained peers (Carmena et al., 2004, Podl et al., 1994). LPL activity in muscles has been reported to increase over 200% in response to intense exercise sessions (Sady et al., 1986), which can be used as indicator of increased post-heparin plasma LPL activity (Podl et al., 1994). The level of LPL post exercise has been investigated in many studies. For instance; Miyashita and Tokuyama demonstrated that 30 min of moderate-intensity cycling performed the day before a meal of moderate fat content reduce postprandial serum TG concentrations but did not affect serum pre-heparin lipoprotein lipase concentrations in young men (Miyashita, 2008). Similarly there was no difference observed in post-heparin plasma LPL activity, although it was correlated significantly with exercise-induced changes in fasting and postprandial TG (Gill et al., 2003).

VLDL is the lipoprotein most influenced by exercise (Peterson et al., 1990, Gill et al., 2001b, Gill et al., 2006). The rise of postprandial lipaemia occurs due to a rise in VLDL remnant apoB-100 particles, not CM or CM remnants or apoB-48 particles (Havel, 2000). Recently it has been demonstrated that the major RLP associated with postprandial hyperlipidaemia is in fact VLDL and not CM remnants (Nakajima et al., 2011a).

Despite the fact that CM is the preferred substrate for LPL (Fisher et al., 1995, Bjorkegren et al., 1997), it seems that VLDL concentration reductions after a session of exercise are mediated by increased peripheral clearance, rather than reduced hepatic production (Al-Shayji et al., 2012). It was hypothesised that this may be due to exercise altering the composition of VLDL₁ to make it a more favourable substrate for LPL (Al-Shayji et al., 2012). **The first objective of this PhD thesis is to explore and understand why LPL clears VLDL₁ more rapidly after exercise by testing the hypothesis that the affinity of VLDL to LPL**

increases after exercise. This work is described in Chapter 3. This may help to improve the understanding of the nature of these exercise-induced changes. For health care professionals this will help to emphasize the reason behind high level of TG and might recommend exercise as part of the treatment for people with high TG level.

1.5.2 Controlling plasma lipids by diet modification

A balanced dietary intake is necessary for optimal health benefits. Despite the essential role of diet in the treatment of many metabolic syndromes, the role of diet in the treatment of hypercholesterolemia has been largely neglected with the advent and use of medications (statins) (Kreisberg and Oberman, 2003). The relationship between plasma lipid and dietary intake has been studied in populations and individuals. Older studies examined total cholesterol and triglyceride levels; more recent studies have looked at specific lipoproteins and apolipoproteins.

Studies relating the effect of diet to serum lipid levels and to rates of atherosclerosis in free-living populations have consisted mostly of cross-sectional surveys. Some studies found correlations between dietary fat and serum lipids when different groups were compared, e.g. Japanese living in urban and rural areas (Ueshima et al., 1982) and Polynesian groups consuming different levels of dietary saturated fat (Prior et al., 1981). Surveys of single populations such as that of the Western Electric Study showed that a high ratio of polyunsaturated to saturated fat was correlated positively with plasma lipid levels and positively with a decreased incidence of coronary heart disease (Shekelle et al., 1981), but the correlation coefficients were small. The Tecumseh Study, on the other hand, failed to show a correlation between fat, cholesterol, and other macronutrient intake and serum cholesterol and triglyceride levels (Nichols et al., 1976). The North American diet pattern of consuming higher quantities of red meat, high-fat dairy products, and refined grains and low amount of fiber was positively correlated with higher CRP (Fung et al., 2001). The Northern Manhattan Study (Gardener et al., 2011), European Prospective Investigation into Cancer and Nutrition (EPIC) cohort reported (Dilis et al., 2012) that a dietary pattern resembling the Mediterranean Diet was inversely associated with a composite

outcome of CVD (ischemic stroke, myocardial infarction or vascular death). Worldwide cross-sectional surveys, both prospective and retrospective, generally support the concept that low-fat diets correlate with lower lipid levels and lower incidence of atherosclerotic heart disease; and they are used to support position papers advocating the adoption of such diets (Gotto Jr et al., 1984). Nevertheless, there are problems in interpreting such studies. It is difficult to assess the part played by other factors such as the type of consumed fat, percentage of fat and carbohydrate, exercise, total caloric intake, obesity, and stress (Gordon et al., 1984). More information concerning the relationships of diets and lipoproteins has come from individual and group feeding studies.

Effect of total fat ingestion on lipid metabolism

There is growing evidence about how major shifts in the macronutrient content of the diet can affect plasma lipoprotein patterns, which can be used to form dietary recommendations to minimize the risk of developing CVD. Early work focused on assessing the effect of a high fat meal on plasma lipoprotein pattern and it was observed that plasma TG increased (Havel, 1957b). However, later in the 1960s, it was noted that diets very low in fat resulted in hypertriglyceridemia (Ahrens Jr et al., 1961, Frayn, 2009, Lichtenstein and Van Horn, 1998). Hudgins and co-worker observed an increase in fatty acid and VLDL-TG induction by very-low-fat, high-sugar diets (Hudgins et al., 1998, Nestel et al., 1970). From the early 1950, the role of fatty acid on plasma cholesterol was studied (Hegsted et al., 1959, Keys et al., 1950) and it is accepted that dietary saturated fatty acids (SFAs) are detrimental to health (Hunter et al., 2010). It was observed that not all saturated fatty acid (SFA) had similar effect on plasma cholesterol. The shorter the chain of saturated fatty acids (6:0-10:0) the lower effect on plasma cholesterol, whereas those with intermediate chain lengths (12:0-16:0) increased concentrations of cholesterol (Keys et al., 1950, McGandy et al., 1970). This increase in cholesterol level has been attributed to a decrease in low-density lipoprotein receptor activity (Matthan et al., 2004). Saturated fatty acid increases plasma LDL-cholesterol concentrations and lowers levels of cholesteryl esters in the liver (Spady and Dietschy, 1985). Myristic and palmitic acids increased LDL-C and HDL-C levels to a similar extent without significantly altering TC/HDL-C ratio, whereas lauric acid had the largest LDL-C- and HDL-C-raising effect leading to a decrease in the

TC/HDL-C ratio (Mensink et al., 2003). Epidemiological studies have found an association between higher saturated fat intake and higher rates of cardiovascular disease (Howard et al., 2006, Dayton et al., 1969). Randomized clinical trials have documented that lowering saturated fat reduces coronary events and reduces cholesterol, and that Mediterranean diet (high in fibre, olive oil, fish, and a 30% fat) improves survival (De Lorgeril et al., 1999, Mauger et al., 2003, Roth and Brown, 2005).

Effect of quality of diet fatty acids on lipid metabolism

Trans-fatty acids

The frequent ingestion of high fat content foods such as margarine, cookies, cake and white bread which contain partially hydrogenated vegetable oils provide the body with *trans*-fatty acid (TFA) which are significantly associated with higher risks of CHD. A diet with the same energy density from saturated or cis unsaturated fats showed that the consumption of trans fatty acids raises levels of LDL-C, reduces levels of HDL-C, and increases the ratio of total cholesterol to HDL cholesterol (Stampfer et al., 1991). For every 1% increase in total calories from TFA, the amount of LDL-C increases by 2% (Cleeman et al., 2001). *Trans* fatty acid also increases the plasma concentration of TG (Mensink et al., 2003), and Lp(a) lipoprotein (Zaloga et al., 2006) and reduces the particle size of LDL-C more than other fats (Mauger et al., 2003). Five weeks consumption of TFA derived partially hydrogenated fats was associated higher total and LDL cholesterol, and lower HDL cholesterol concentrations in post-menopausal women (Matthan et al., 2004). These changes were attributed to impaired LDL apoB-100 and enhanced HDL apoA-I fractional catabolism. Consumption of TFA did not alter TRL apoB-100 or apoB-48 metabolism (Matthan et al., 2004). The combined effect of dietary saturated and *trans* fatty acids on plasma lipids is amplified by the lack of n-3 long-chain fatty acids, which have complex competitive effects on prostanoid synthesis, cellular function, and thrombosis (Amine et al., 2002). Epidemiological studies have demonstrated that diets low in saturated fat are associated with lower rates of CVD (Vessby, 2003, Trial, 2006). For instance the inverse relationship of dietary ω -3 fatty acids and CVD events (Dyerberg et al., 1975). However, The effects of

dietary fats on plasma TG is less clear than the effects of dietary fats on cholesterol and tend to be determined by specific fatty acids (Howell et al., 1997).

Monounsaturated fatty acid

Zheng *et al.* reported that replacing 17% of total energy intake from complex carbohydrate with MUFA selectively stimulated the secretion of VLDL and IDL particles containing apoE and apoC-III, while suppressing the secretion of particles that did not (Zheng et al., 2008). Consequently, the concentration of apoB-containing lipoproteins with apoE and apoC-III was higher with the MUFA diet compared with the carbohydrate diet (Zheng et al., 2008). The MUFA diet was also associated with increased VLDL and IDL apoB catabolism, although LDL apoB metabolism was not altered (Zheng et al., 2008). This study lends support to the potential benefits of increased MUFA and reduced complex carbohydrate in modulating lipoprotein metabolism. The study, however, was non-randomized and short-term, with an intervention period of only three weeks. Notably, this study compared two relatively healthy diets, both low in SFA, high in fiber and utilized primarily low glycaemic foods. High-MUFA diets have gained significant attention as an alternative dietary pattern to the commonly recommended low-fat and high-carbohydrate (CHO) pattern. Several meta-analyses of randomized controlled trials (RCTs) suggested potential benefits of a high-MUFA diet compared with a high-CHO diet in improving metabolic factors, such as glycemic control, serum lipids, and blood pressure, among both healthy individuals and T2D patients (Schwingshackl et al., 2011, Shah et al., 2007, Garg et al., 1994). A recent meta-analysis illustrated that, When comparing high-MUFA to high-CHO diets, there were significant reductions in fasting plasma glucose, TG, body weight, and systolic blood pressure along with significant increases in HDL cholesterol (Qian et al., 2016). Also a diet rich in MUFA has been linked to a reduction in LDL oxidation. Studies found that the particles rich in MUFA have been shown to be less susceptible to oxidative modification compared to LDL particles enriched with n-6 fatty acids (Parthasarathy et al., 1990, Abbey et al., 1993, Bonanome et al., 1991).

Polyunsaturated fatty acids

An aspect of CVD research focuses on the cardio-protective effects of fish oils and of individual omega 3 polyunsaturated fatty acids (n-3 PUFA), or more specifically, eicosapentaenoic acid (EPA; 20:5 n-3), docosahexaenoic acid (DHA; 22:6 n-3) and α linolenic acid (ALA; 18:3 n-3). Many large-scale studies, including primary and secondary prevention clinical trials and meta-analysis of cohorts, have concluded that consumption of fatty fish, fish oils or individual n-3 PUFA is an effective dietary strategy to lower CVD morbidity, mortality, as well as classic and emerging risk factors (Bucher et al., 2002, von Schacky, 2003, Calder, 2004, Von Schacky and Harris, 2007, Yaktine and Nesheim, 2007, Hu et al., 2002). Clinical and epidemiological trials have shown that an elevated intake of long-chain was also associated with a reduced cardiovascular risk, reduced inflammatory processes and reduced all-cause mortality (Simopoulos, 2008, Tapiero et al., 2002). A high intake of n-3 PUFA has been associated with antiarrhythmic, anti-thrombotic and vasodilatory effects (De Caterina, 2011, Leaf et al., 2003). Replacement of energy from saturated fat with PUFA has been shown to decrease TC and LDL-C, with a concomitant decrease in HDL-C (Hodson et al., 2001). In addition, n-3 PUFA have been shown to improve a number of cardiac hemodynamic factors such as blood pressure (Mozaffarian et al., 2006, Geleijnse et al., 2002) and endothelial function (Hirafuji et al., 2003, Goodfellow et al., 2000) plasma TG reduction (Harris, 1997) anti-inflammatory responses (Calder, 2006) and anti-atherosclerotic effects (von Schacky, 2003).

Effect of carbohydrate ingestion on lipid metabolism

On the other hand, it has been observed that the ingestion of CHO alone (as in an oral glucose tolerance test (OGTT)) has no influence on lipoprotein particles (Havel, 1957a). In free living, the majority of ingested food contains CHO, fat and protein. Most human interventions are carried out by holding the protein content of the diet relatively constant and varying the amount of fat and CHO. Therefore, many studies have been examining the effect of different ratios of fat/CHO diets on metabolism. Increases in the relative proportion of CHO result in reductions in HDL-C and an increase in TG concentration (Stampfer et al., 1996, Lichtenstein et al., 1994, Grundy et al., 1986, Mensink et al., 2003).

Diets high in carbohydrate (60-80% of calories) and low in fats (0-25% of calories) have marked effects on plasma levels of lipoproteins and on lipoprotein metabolism. The substitution of SFA by CHO decreases HDL-C and increases TG (Knopp et al., 2000, Howard and Wylie-Rosett, 2002). Plasma TG and VLDL-TG rise in both normal and hypertriglyceridaemic subjects (Blum et al., 1977, Falko et al., 1980b, Falko et al., 1980a, Ginsberg et al., 1981, Huff and Nestel, 1982, Kashyap et al., 1982, Liu et al., 1984, Schonfeld et al., 1976). Hepatic secretion of VLDL increases (Schonfeld and Pflieger, 1971), and the VLDL becomes more TG enriched (Falko et al., 1980b, Ginsberg et al., 1981, Kashyap et al., 1982). The elevations in plasma TG reach a peak after one week and decrease after three weeks, although they do not return to baseline levels in that time (Kashyap et al., 1982). Interventional studies comparing the effects of low and high carbohydrate diets on LDL found that high-carbohydrate diets affect LDL particle size, generating smaller, potentially more atherogenic LDL particles as compared to low carbohydrate diets. During 3 days. After the low-fat and high-carbohydrate diet, LDL particle size distribution shifted towards smaller particle size. These changes in LDL particle size were thought to be the consequence of an observed increase in large triglyceride-rich VLDL particles and serum triglycerides, which both are of importance in the generation of small, dense LDL as mentioned above (Guay et al., 2012). However, changes such as those observed in the above-mentioned study have not been observed in another study after just a single meal with different fat content (Callow et al., 2002). One study of 4 weeks duration demonstrated a smaller LDL peak size in individuals after a low-fat and high-carbohydrate diet compared to a high-fat and low-carbohydrate diet (Faghihnia et al., 2010). A long-term diet intervention over 9 months has been performed in overweight or obese middle-aged adults. Most importantly, LDL size increased in this population during a low-carbohydrate diet, whereas no differences were observed during a low-fat diet. The change in body weight did not differ between these two groups (LeCheminant et al., 2010). HDL-C also decreases after a few days on a high - carbohydrate diet (Kashyap et al., 1982, Gonen et al., 1981, Blum et al., 1977, Schonfeld et al., 1976), with the HDL₂ fraction falling to a greater extent than HDL₃ (Gonen et al., 1981). Thus, in some respects diets high in carbohydrate and those with high P/S ratios both produce similar effects on LDL and HDL, but not on VLDL. There are significant changes in apolipoprotein concentrations in

response to these short-term increases in dietary carbohydrate. Proteins of the C family increase in response to high-carbohydrate diets. Plasma levels of apoC-II increase both in normal subjects fed a high-carbohydrate formula diet and in patients with high plasma TG (Falko et al., 1980b). Kashyap et al, found increases in both apoC-II and C-III but the ratio of apoC-III to apoC-II was lower in VLDL and HDL₂ after three weeks of a high-carbohydrate diet; this indicates a relative enrichment of VLDL and HDL₂ with apoC-II. Kashyap et al suggest that the difference in amounts of apoC-III and C-II in HDL₂ subfractions may be due to differences in binding affinities of the different apoC's for HDL subfractions (Kashyap et al., 1982).

The type of carbohydrate used in the diet significantly affects TG and VLDL responses. On a diet with 70% carbohydrate and with sucrose contributing 55% of calories, VLDL increased in six of seven subjects (Nestel et al., 1979). VLDL-apoB also increased. In two subjects there were increases in removal rates of apoB VLDL from the circulation, whereas in four subjects there was decreased removal of apoB VLDL. In most of the studies involving high carbohydrate diets, sucrose comprised a large part of the additional carbohydrate calories. In a recent study of hypertriglyceridaemic subjects, when the sucrose content of the diet was increased from 9 to 15% while total carbohydrate content was increased from 40 to 60%, the degree of fasting hypertriglyceridemia and increase in VLDL triglyceride were greater than if sucrose was kept at a constant percentage of total carbohydrate (Liu et al., 1984). VLDL triglyceride, VLDL cholesterol, and VLDL protein rise by factors of 2.4, 1.67, and 1.88 respectively; LDL cholesterol decreases while LDL triglyceride remains the same. However, this response is attenuated when complex CHO primarily consisting of whole grains are given. Fifty percent CHO intake worsens lipid and non-lipid risk factors in patients with the metabolic syndrome. However, some CHO commonly referred to as complex has a higher glycaemic index (GI) and consequently increase insulin responses more than simple sugars (Ludwig, 2002). GI is a term used to classify food according their effects on glycaemic responses (Wolever et al., 1991). High GI Foods are digested and transformed into glucose rapidly for a given amount of CHO than foods with a low GI (Axen and Axen, 2010, Rossetti et al., 1987). A high glycaemic load (product of GI and CHO content) is associated with higher fasting TG and lower HDL-C levels

(Ludwig, 2002). Increased dietary carbohydrates, particularly simple sugars and starches with high glycemic index, can increase levels of small, dense LDL (Siri and Krauss, 2005). Ingesting high glycaemic meals can lead to various metabolic changes that promote excessive food intake (Ludwig et al., 1999). On the other hand, a meta-analysis of observational studies reported an association between the consumption of lower GI foods and lower TG, LDL-C and total cholesterol and higher HDL-C concentrations (Barclay et al., 2008, Goff et al., 2013). It was estimated that replacement of high GI foods for low GI foods can result 15% to 25% reductions in TG concentrations (Pelkman, 2001).

However, most studies have examined the long-term effect of high CHO diet or high fat diet or specific fatty acid ingestion on total plasma TG, HDL and LDL. There is limited data on the acute effect of fat, CHO or combination of both on the lipoprotein particles metabolism.

Effects of dietary fibre on lipid metabolism

Dietary fibre and whole grains contain a unique blend of bioactive components including resistant starches, vitamins, minerals, phytochemicals and antioxidants. As a result, research regarding their potential health benefits has received considerable attention in the last several decades. Epidemiological and clinical studies demonstrate that consumption of dietary fiber and whole grain intake is inversely related to obesity (Liu et al., 2002), type two diabetes (Meyer et al., 2000), cancer (Forman and Hernandez, 2010) and CVD (Streppel et al., 2008).

Dietary fibre, by its impact on the glycaemic response and other aspects of metabolism, may also have important effects on cardio metabolic pathways (Pereira and Liu, 2003). An increasing number of studies have reported that for every 10 g of additional fiber added to a diet the mortality risk of CHD decreased by 17-35% (Pereira et al., 2004, Streppel et al., 2008). Based on findings from epidemiologic studies regarding the protective effects of fiber intakes, the Dietary Reference Intakes (DRI) recommended consumption of dietary fiber is 14 g/1000 kcal, or 25 and 38 g/day for adult women and men, respectively (Slavin, 2008).

It has been proposed that dietary fibre could modify underlying CVD risk factors including lipid and lipoprotein metabolism, insulin homeostasis, inflammatory markers and coagulation, and improves insulin sensitivity, thereby reducing the

risk of CVD mortality (Erkkilä and Lichtenstein, 2006, Kokubo et al., 2011, Eshak et al., 2010). Although studies showed beneficial effects of soluble, gel-forming fiber on cardio metabolic risk factors, food sources of mainly insoluble fibers, primarily contributed by cereal products, have been the fiber most consistently associated with lower risk of CVD (Erkkilä and Lichtenstein, 2006). Findings of some investigations also suggest that the role of dietary fibre is more dependent on its types and sources, rather than the amount of intake (McKeown et al., 2004, Hosseinpour-Niazi et al., 2011). Different types or sources of dietary fibre may induce different physiological effects; soluble fibre is responsible for the cholesterol-lowering effect of dietary fibre whereas insoluble fiber interacts with intestinal absorption of foods and contributes to reduction in clotting factors (Brown et al., 1999).

It has been demonstrated that inflammation may be an important mediator in the association between consumption of dietary fibre and CVD. A research has demonstrated an association between dietary fibre and levels of C-reactive protein (CRP), a clinical indicator of inflammation (King et al., 2003). After adjustment for age, gender, race, education, smoking, physical activity, BMI, total energy consumed, and fat intake. Dietary fiber intake from a variety of sources has been associated with a significantly decreased risk of coronary heart disease. In the Nurses' Health Study, women in the highest quintile of fibre intake (median 22.9 g/day) had an age- adjusted relative risk for major coronary events that was 47% lower than women in the lowest quintile (11.5 g/day) (Stampfer et al., 2000).

Several studies have evaluated dietary fibre in relation to intermediate vascular markers such as cholesterol. Studies suggest that 3 g soluble fiber from oats (three servings of oatmeal, 28 g each) can decrease total cholesterol and LDL by 0.13 mmol.l⁻¹ (Gulati et al., 2017, Katz, 2001). The mechanism can be explained by the reduction in the absorption of ileal bile acid (Morgan et al., 1993). These soluble forms of dietary fibre appear to have a negligible effect on triglyceride or HDL levels. Insoluble dietary fibre subgroups are derived largely from cereal sources and have little or no effect on lipids and lipoproteins (Jenkins et al., 2000).

Effect of high fat vs. high carbohydrate diets

In a meta-analysis of randomized controlled trials comparing high fat diets with high carbohydrate diets, it has been found that, both diets reduced participants'

blood pressures, total to HDL cholesterol ratios, and total cholesterol, LDL cholesterol, triglycerides, blood glucose, and serum insulin levels and raised HDL cholesterol; however, participants on high fat diets had greater increases in HDL cholesterol and greater decreases in triglycerides but experienced less reduction in total and LDL cholesterol compared with persons on high carbohydrate diets (Hu et al., 2012, Mansoor et al., 2016b, Sackner-Bernstein et al., 2015). However, it should be noted that the associations between diets and lipid profiles may be stronger in shorter-term studies due to greater control over the participants' diets and higher compliance rates, and might provide insight to how these effects might be long-term. The acute effect of high carbohydrate diet on lipid metabolism would explain the mechanism behind the long term ingestion. It has been shown that ingesting high carbohydrate diet by patients with non-insulin-dependent diabetes mellitus led to little or no decrease in postprandial plasma or lipoprotein TG or cholesterol concentrations and an actual increase in concentration of potentially atherogenic small chylomicron and/or chylomicron remnants (Chen et al., 1992). Also, in nondiabetic population, it reduces total, LDL and HDL cholesterol. In contrast, the acute ingestion of fat has been related to increase in the postprandial concentrations of TG, phospholipid and remnant lipoprotein (Wilson et al., 1985, Nestel, 1964, Jeppesen et al., 1995, Cohen et al., 1988, Havel, 1957b). In addition, the acute fat ingestion increases plasma NEFA concentrations (Katan et al., 1994); also, the acute ingestion of fat led to stepwise increases in the postprandial rise of chylomicron and serum TG and induced marked changes in serum lipoproteins postprandially (Dubois et al., 1998). However, there is a limited knowledge in the effect of the acute ingesting equal amount of carbohydrate with fat or ingesting them separately on both TRL and CRL (Austin, 1990).

In real life, fat and carbohydrate are consumed together, thus it is important to understand how this co-ingestion influences postprandial metabolic responses. Manipulating dietary macronutrient content has been shown to improve blood lipid profiles even in the absence of weight loss. For example, high fat diet increased HDL concentrations and decrease TG concentrations (Shai et al., 2008). Another study that compared the effects of dietary macronutrient content, found that the high fat/low carbohydrate diet resulted in greater improvements in blood lipids

and systemic inflammation when compared to the low fat/high carbohydrate, even though there were no detectable differences in body weight between the two diets (Ruth et al., 2013). On the other hand, high carbohydrate diets are linked to the elevation of fasting plasma TG concentrations (Parks et al., 1999, Roche, 1999), small but significant increases in total, LDL and HDL cholesterol (Wood et al., 2016, Mansoor et al., 2016a, Bueno et al., 2013, Hu et al., 2012), and higher postprandial glucose and insulin concentrations (Mohammed and Wolever, 2004). Ultimately, this may decrease insulin sensitivity (Wolever and Mehling, 2003), raising fasting triacylglycerol concentrations but also induced a shift toward smaller, denser LDL particles (Krauss, 2001).

Therefore, the aim of the second experimental Chapter 4) is to determine the influence of the ingestion of CHO with fat on lipoprotein metabolism which will provide the information about acute ingestion of fat and carbohydrate on lipoprotein metabolism.

1.6 Ethnicity, plasma lipids and postprandial response

Environmental, genetic and lifestyle factors are thought to affect the distribution, incidence and mortality of CVD and diabetes among racial and ethnic populations (Haga and Venter, 2003, Cooper, 1997a, McBean et al., 2004). Ethnicity play important roles in understanding disparities in health and health care (Karter, 2003a, Karter, 2003b). Ethnicity has been defined as “a complex multidimensional construct reflecting the confluence of biological factors and geographical origins, culture, economic, political and legal factors, as well as racism” (Williams, 1997).

1.6.1 Ethnicity and plasma lipids

As mentioned above there are ethnic differences in the incidence of CVD. This can be as a result of differences in lipid profile and/ or differences on CVD risk factors. Differences in the lipid and lipoprotein concentrations between African American (AA) and Caucasian (CA) populations have been reported to occur in children (Crawford et al., 2001, Williams et al., 1992, Berenson et al., 1981, Morrison et al., 1979, Donahue et al., 1989) and this continues till adulthood (Tyroler et al., 1975, Srinivasan et al., 1986, Tyroler et al., 1980). These differences include lower plasma TG and VLDL-cholesterol, as well as higher levels of HDL cholesterol in AA compared with CA women (Tyroler et al., 1975, Srinivasan et al., 1986, Tyroler et al., 1980). These differences in the plasma lipids and lipoprotein concentrations persist after adjustment for age, degree of obesity and adiposity, and the use of tobacco and alcohol (Tyroler et al., 1980, Glueck et al., 1984). This is also had been reported in black men when compared to White Europeans, Blacks generally have a favourable lipoprotein-lipid profile including low fasting TG and apoB levels and higher HDL-C concentration (Després et al., 2000, Lovejoy et al., 1996, Albu et al., 1997, Howard et al., 2003, Kuller, 2004, Chaturvedi et al., 1994). Similarly, Japanese have higher HDL-C and TG levels than Whites (Wolfe et al., 2002). Afro- Caribbean men have lower levels of LDL-C, total cholesterol, TG, large and small VLDL, sdLDL, as well as lower VLDL particle size, than either African American or White men (Miljkovic-Gacic, 2006). On the other hand, South Asians have high incidence of CHD and more than double risk of developing T2D

than Whites, Hispanics and Blacks. This might be related to increased frequency of IR and diabetes, elevated TG and low HDL-C in Asians (Anand et al., 2003, McKeigue et al., 1991, Chandalia et al., 2007). Middle Eastern (ME) adults seem to have mean plasma cholesterol levels of $\sim 5.2 \text{ mmol.l}^{-1}$ (Al-Nozha et al., 2008, Al-Lawati et al., 2003, Zindah et al., 2008), which is near to the average European level 5.0 mmol.l^{-1} (WHO, 2014a).

A possible explanation of these variations is low birth weight, In a retrospective study of adult men and women living in South India, the prevalence of CHD was related to their size at birth, low birthweight, short birth length and small head circumference at birth and these were all associated with a raised prevalence of CHD later in life (Stein et al., 1996)=. In Jamaican schoolchildren age between 6-12 years, indices of small size at birth were associated with higher systolic BP, glycated haemoglobin and serum cholesterol levels (Forrester et al., 1996).

Another possible explanation for differences are alterations in the metabolic factors that regulate lipoprotein levels. For example, the activity of LPL in subcutaneous adipose tissue of lean black African males residing in the Quebec City area no more than 3 years is higher than lean Caucasian males (Ama et al., 1985). Also it has been found that the rate of clearance of TG from circulation was higher in the AA men. Accompanying this increased clearance was an elevation in post-heparin plasma LPL activity (Friday et al., 1999). Other alterations in metabolism are seen in hyperinsulinaemic South Asians where there is, raised TG and lower HDL cholesterol levels, central adiposity with a high waist to hip ratio (McKeigue, 1991, McKeigue et al., 1992, Cruickshank et al., 1991). Moreover, Black subjects both men and women, have significantly higher post heparin hepatic lipase activity but lower hepatic lipase activity than White subjects (Albu et al., 1997). The low hepatic lipase activities in Blacks may have a genetic basis and contribute to their higher plasma HDL-C concentrations when compared to Whites (Vega et al., 1998).

1.6.2 Ethnicity, obesity and adipose tissue

The distribution of overweight and obesity in this population is known to vary considerably by ethnic group (Saxena et al., 2004). For the same BMI as

Caucasians, the body fat percentage in Asians would be 5-7% higher in Indian men (Forouhi et al., 1999, Nair et al., 2008, Chandalia et al., 2007) 8% in Indian women (Rush et al., 2009, Rush et al., 2007, Kamath et al., 1999) 1-4% in Japanese women (Gallagher et al., 2000), 5% and 7% for Indonesian men and women from Malay ancestry respectively (Gurrici et al., 1999), and 1.3% and 1.7% for Indonesian Chinese men and women respectively (Gurrici et al., 1999). Interestingly, there was a tendency that the difference in body fat percentage became smaller with increasing BMI and age (Gallagher et al., 2000). In Asians, it was predicted that with increasing age, the body fat percentage increased to a lesser degree than in Caucasians (Chung et al., 2005, Rush et al., 2009). Among Asians, Indians have the highest body fat percentage followed by Malays and Chinese. The suggested BMI cut-off points for obesity are 26 kg.m^{-2} for Indians, 27 kg.m^{-2} for Malays and 27.5 kg.m^{-2} for Chinese, as compared to 30 kg.m^{-2} for Caucasians (Deurenberg-Yap et al., 2001). It can be concluded that the difference in body fat percentage between Asians and Caucasians is dependent on the region/ethnicity. It is most pronounced in South (Indians), than Southeast (Malay) and than East Asian (Chinese/Japanese). Ethnic differences in the relationship between BMI and body fat percentage may be explained by the difference in body build and frame size (Deurenberg et al., 1999), in part by differences in muscularity and bone mineral content (Rush et al., 2007) as well as fat distribution and relative leg length (Rush et al., 2009).

Ethnicity also influences adipose tissue distribution. For a similar level of total body fat, white subjects have been shown to have more intra-abdominal adipose tissue than Blacks (Despres, 2000, Hoffman et al., 2005). It has been suggested that Asian, Hispanic and Caucasian are particularly prone to intra-abdominal fat and associated health risks. Interestingly, besides having less intra-abdominal adipose tissue accumulation, Black women are more likely to develop IR. This finding holds up even after matching them with White women for age, degree of obesity, and waist-to-hip ratio (Lovejoy et al., 1996). However, it was suggested that Black individuals are less likely to have intra-abdominal fat deposition and therefore have a more favourable metabolic profile than White individuals (Despres, 2000, Albu, 1999). Similarly, in Nurses' Health Study, the increases in weight over 20 years were more harmful in Asians than other ethnic groups. For

every 11 pounds weight gained during adulthood, Asians had 84% increased risk of T2D. The risk for diabetes with weight gain also increased for Hispanics and Blacks but to a much lesser degree than Asians (Deurenberg-Yap et al., 2000, Wen et al., 2009, Pan et al., 2004). The possible explanation for this could be amount of body fat.

Interestingly, Far-East Asians and South Asian are different from Caucasians and each other in BMI and body fat percent relationship (Pan et al., 2004, Deurenberg-Yap et al., 2000). Despite lower BMI indices, Asian populations have an increased tendency to accumulate Intra-abdominal Fat (IAF) and are more likely to develop T2D and CVD (Abate et al., 2001, Ramachandran et al., 2006, Chandalia et al., 2007). South Asians, in particular, have high levels of body fat and are more prone to develop abdominal obesity, which may account for very high risk of T2D and CVD (Misra and Khurana, 2009, Misra and Vikram, 2004). Additionally, several studies have shown that Japanese Americans have a greater amount of intra-abdominal adipose tissue and higher prevalence of T2D (Boyko et al., 1995, Fujimoto et al., 1999). A 32% of adults in the Middle East are having a BMI ≥ 30 kg.m⁻² (WHO, 2014b). Unfortunately, there is no available data of fat distribution among the Middle Eastern.

Obesity, particularly visceral adiposity, is associated with IR and often assumed to be causative. According to the “lipid supply” hypothesis, higher concentrations of FA resulting from higher fat intake or higher visceral or intramuscular fat, inhibit carbohydrate oxidation and thereby produce IR (Kraegen and Cooney, 1999). However, the Arabic Caucasian subjects had the highest measure of abdominal fat (using WHR) but were among the most insulin sensitive (Dickinson et al., 2002). Furthermore, the Arabic Caucasian group reported the greatest fat intake (36% of energy vs. only 28% in the European Caucasian group), but the two groups were indistinguishable on the basis of HOMA-IR or postprandial responses to the bread meal. Hence differences in “lipid supply” do not account for differences in insulin sensitivity among these young adults (Dickinson et al., 2002).

1.6.3 Ethnicity and diabetes

The West Pacific region had the highest number of individuals (131.9 million) diagnosed with diabetes with a comparative prevalence rate of 8.3%. The two countries in this region that had the highest prevalence rates were the Kiribati and Marshall Islands, with rates of 25.7% and 22.2%, respectively. Middle East and North Africa regions had the highest comparative prevalence rates of diabetes at 11.3%. Six countries in this region are among the world's top ten countries for highest diabetes prevalence rates, Kuwait (21.1%), Lebanon (20.2%), Qatar (20.2%), Saudi Arabia (20.0%), Bahrain (19.9%) and United Arab Emirates (19.2%). The North America/Caribbean region had the second highest comparative prevalence rate of diabetes at 10.7%. South-Central America and South East Asia had similar diabetes prevalence rates of 9.2%, whereas Europe had a 6.7% comparative prevalence rate. Africa had the lowest comparative prevalence rate of diabetes (4.5%); however the Africa region has the highest proportion of undiagnosed diabetes, with at least 78% of affected individuals being undiagnosed (Cho et al., 2013, International Obesity Task Force 2012). Pima Indians and Asian Indians are more insulin resistant than European Caucasians of similar age and BMI (Chandalia et al., 1999, Lillioja et al., 1991). In most studies, however, the subjects have been middle-aged and/or overweight, with IR already well developed. Studies in young lean subjects are required to determine whether reduced insulin sensitivity and postprandial hyperglycemia/hyperinsulinemia can be present without overt signs of the metabolic syndrome.

Although the different explanations mentioned above there is a difference in the metabolic response in different ethnic groups. The glycaemic response seems to favour White Europeans compared with South Asians (Mohan et al., 1986, Raji et al., 2001). Also, it has been observed that higher insulin concentrations after glucose load were reported in both normal and diabetic Navajo Indians compared with Pennsylvania Amish of similar weight (Rimoin, 1969). It has been observed that stimulated plasma insulin concentrations in the Pima Indians were 2-3 times higher than in the White population (Aronoff et al., 1977). The Pima Indians of the Gila River Indian Community in Arizona, a population in whom insulin-dependent diabetes is unknown, have the world's highest reported incidence of NIDDM, which often occurs in early adulthood (Savage et al., 1979, Knowler et al., 1978). Afro-

Caribbean also, have a higher insulin response compared with Whites (Chaturvedi et al., 1994). It has been reported, in lean, young adults of Thai, Vietnamese and Chinese origin displayed marked postprandial hyperglycemia and hyperinsulinemia compared with matched Caucasian subjects. This corresponded to reduced insulin sensitivity as determined by HOMA, although the level of insulin sensitivity was still within the normal range for healthy individuals. The differences among ethnic groups were evident despite similarities in age (mean 20-22 y), BMI (20-23 kg.m⁻²), waist circumference (72-82 cm), birth weight (3.2-3.5 kg) and diet (Liew et al., 2003). After a high fat meal consist of (52% of energy as fat, 40% as carbohydrate and 8% as protein), the postprandial lipaemia is not affected in young South Asians compared to Northern Europeans although glucose intolerance is detectable (Cruz et al., 2001a). Consuming a carbohydrate meal that's provides 75 g of available carbohydrate, it has been found that SE Asians had the highest postprandial glycaemia and lowest insulin sensitivity, whereas European and Arabic Caucasian subjects were the most insulin sensitive and carbohydrate tolerant (Dickinson et al., 2002). Also, the lipaemic response after a high liquid fatty meal (86.5% of calories from fat), The increase in TG levels at 2 and 4 hours postprandially, tended to be lower in the AA than the CA (Bower et al., 2002).

1.6.4 Ethnicity and other CVD risk factors

Another possible cause of the variability of CVD among different ethnic groups could be due to the prevalence of risk factors such as hypertension. It has been observed that in both genders in Black population tend to have higher prevalence of hypertension than Whites (Greenlund et al., 1998). In addition, family history of hypertension was also greater among Blacks than Whites within sites. Alvis et al reported significantly higher age adjusted CVD mortality rates among Black men with hypertension (65.1 per 10,000 person/years) compared to Whites (55.8 per 10,000 person/years) (Thomas et al., 2005). Other factors can influence ethnic differences such as environmental factors with respect to access to healthy food sources, places to exercise or crime related safety (Golden et al., 2012). Lack of healthy food stores, lack of places to exercise and increased psychosocial stressors related to crime or limited social cohesion have been linked to poor health outcomes (Casagrande et al., 2009). Evidence from the Multi-Ethnic Study of Atherosclerosis found that better neighbourhoods were associated with improved

insulin sensitivity and decreased risk of type 2 diabetes (Auchincloss et al., 2009). Neighbourhoods have also been associated with increased smoking, physical inactivity and poorer control of blood pressure, which can contribute to development of diabetes and its complications (Gary et al., 2008). For instance, Mexican American women had the highest prevalence of no leisure-time physical activity compared to Black and White women (Winkleby et al., 1998). American Indian and Alaska Natives (AIANs) (Denny et al., 2005) and Blacks (Appel et al., 2002) also had higher prevalence of no leisure-time physical activity compared to Whites. Management of chronic diseases can also be more difficult in low socioeconomic areas (Brown et al., 2007). A study conducted in the GCC countries revealed that only 40% of men and 27% of women reported that they were physically active for at least 150 min per week (Mabry et al., 2010).

Smoking also varies between ethnic groups, non-Hispanic Black (NHB) and non-Hispanic White (NHW) have been reported to have similar smoking rates whereas Native Americans and Alaska Natives have higher smoking rates compared to NHB and NHW. Mexican Americans have the lowest smoking rates (Kurian and Cardarelli, 2007). In the Asian population there is great variability in the rates of smoking with the highest rates among Korean men and the lowest among the Asian Indian men (Venkat Narayan et al., 2010). Higher smoking rates among Native Americans may explain the higher prevalence of diabetes and peripheral arterial disease in this population. The rates of cigarette smoking in the GCC ranged from 13.4% to 37.4% in males and from 0.5% to 20.7% in females. Furthermore, the prevalence of smoking fluctuated from age group to age group. It was more common in males at younger ages (18-25 years); however some studies reported a high prevalence in the older age group (40-59 years). In females, the highest rates of smoking were in the older age group (40-49 years) (Bassiony, 2009, Hajat et al., 2012, Memon et al., 2000, Hamadeh and Musaiger, 2000).

1.6.5 Middle East and North Africa

The most frequent comparisons of ethnic groups have been between Caucasian vs. Asian and Caucasian vs. African American. This is because most of the studies were carried out in the UK and US where these are the dominant ethnic groups. Other

countries are now undertaking similar investigations therefore other populations are increasingly subject to study.

For example, in the last 10 years, there have been reports of high prevalence of haemoglobin disorders, neurogenetic disorders, birth defects and inherited metabolic diseases in Middle Eastern and North African populations (Teebi, 2010, Al-Gazali et al., 2006, Christianson et al., 2006). For example, as mentioned in previous sections there has been an increase in the prevalence of non-communicable diseases such as, heart disease by around 44%, stroke 35%, and diabetes 87% (International Diabetes Federation, 2014, WHO, 2014a).

The populations of The Middle East and North Africa (MENA) belong to a unique genetic pool because of the high historical rates of horizontal mixing between ethnicities, the high rate of consanguineous marriages within subpopulations, and the broad geography of the states making up the region. Nevertheless, wide prospective population studies on the effects of polymorphisms on such disorders of metabolism in the MENA are still lacking (Farhud and Yeganeh, 2010). It will be helpful to understand the metabolic response for this population. Therefore, it is timely to investigate the impact of ethnicity on the postprandial metabolism on lipoproteins, glucose and insulin.

Therefore, the aim of the third experimental chapter (Chapter 5) is to illustrate the differences in lipid metabolism, glucose and insulin after ingesting a combined meal, between European and Middle Eastern men.

1.7 Aim of the thesis

The aims of this thesis are:

- 1) Determine the effect of moderate exercise on the affinity of triglyceride rich lipoproteins to lipoprotein lipase
- 2) Examine the effect of the co-ingestion of fat with carbohydrate on lipoprotein metabolism.
- 3) Compare the postprandial response of middle-aged European men and Middle Eastern men.

2 General methods

2.1 Subject Recruitment and Screening

Subjects were recruited from the population in the Glasgow area via local advertising. All subjects were required to attend a screening visit at the university prior to participation to ensure they met with the inclusion criteria of each study. They were provided with an information sheet describing the aim of the study, the experimental procedures involved and any potential risk or discomfort associated with these procedures. Written, informed consent was recorded for each subject (**Appendix A1** for the study in Chapter 4 and **A2** for study in Chapter 5). Questionnaires detailing the subject's past and present health status and family history of disease were completed (**Appendix B**). Resting blood pressure was measured at screening using an automated sphygmomanometer (Omron Healthcare, Inc., Illinois, USA) and fasting finger-prick blood samples were taken to determine glucose using Accu-Chek Aviva Blood Glucose® instrument and Accu-Chek Aviva® Test reagent strips. Once the analysis finished, each participants received a feedback sheet (**appendix D**).

Exclusion criteria common to all studies were:

- A history of known CVD (e.g myocardial infarction, stroke, coronary artery bypass graft surgery), acute illness, or active, chronic systemic disease.
- Anaemia ($\text{Hb} < 12 \text{g.dl}^{-1}$).
- Diabetes (fasting blood glucose $\geq 7 \text{ mmol.l}^{-1}$).
- Abnormal renal, liver or thyroid function tests.
- Participation in a recent research study within last three months.

2.1.1 Anthropometric Measurements

Standing Height

Stretch stature method was used to measure the height using a stadiometer (Seca, Hamburg, Germany). Stature is the maximum distance from the floor to the highest point of the skull when the head is held in the Frankfort plane position (Ross and Marfell-Jones, 1991).

Height was recorded to the nearest 0.1 cm.

Body Mass

Subjects were asked to wear light and minimal clothing and to remove their shoes. Weight was measured using a balanced beam scale to the nearest 0.01 kg. BMI was then calculated as body mass in kilograms divided by the square of height in metres.

Waist and hip circumference Measurement

Waist and hip circumference were measured using a flexible, steel tape measure (Supralip®160, West Germany) in direct contact with the skin. Waist circumference was taken with subjects standing with feet shoulder-width apart and arms on the side and landmarked as the narrowest part of the torso, mid-way between the inferior margin of lowest rib and the iliac crest with the abdominal muscles relaxed. Hip circumference was taken with the subjects standing with feet together and arms at the side and landmarked as the maximum circumference over the trochanters (buttocks) (Lean et al., 1995). The tape was placed horizontally directly on the skin with respect to both landmarks. All measurements were taken at the end of a normal expiration, with repeat measurements. Two measurements were made and the average was taken. If the measurements differed by more than 0.5 cm, a third measurement was made.

2.1.2 Skinfold Measurement

A skinfold thickness is defined as the measure of the double thickness of the epidermis, underlying fascia and subcutaneous adipose tissue on different standard anatomical sites around the body. The following four sites were used according to Durnin and Womersley (1974) who validated the sum of four skinfold thickness against densitometry and devised sex- and age-dependent population-based linear regression equations to estimate total body density (Durnin and Womersley, 1974):

- 1) Biceps: vertical skinfold raised on the anterior aspect of the biceps;
- 2) Triceps: vertical skinfold raised on the posterior aspect of the triceps, mid-way between the olecranon process and the acromion process (shoulder) when the hand is supinated;
- 3) Subscapular: oblique skinfold raised 1 cm below the under most tip of the inferior angle of the scapula at approximately 45° to the horizontal plane following the natural cleavage lines of the skin;
- 4) Suprailiac: diagonal fold raised immediately superior the crest of the ilium on a vertical line from the mid-axillary line.

Skinfold sites were landmarked on the body prior to measurement so that repeat measures could be taken at the same place. The skin at each respective site was pinched up firmly between thumb and forefinger to raise a double layer of skin and the underlying adipose tissue, excluding the muscle tissue. The calipers were then applied to the fold with 1 cm between the edge of fingers and the nearest edge of the calliper and a reading in millimetres (mm) was recorded. All skinfold measures were taken on the right side of the body with skinfold calliper (Holtain Ltd., Crymych, UK). Measurements were recorded in duplicate for each site, not taken consecutively but by running through all sites once and back again as to allow the skin to regroup between measurements. If the readings for each site were more than 5% apart, a third measurement would then be taken, and the two closest measurements were

taken for calculation. The sum of the four skinfolds ($\Sigma 4SF$ = biceps + triceps + subscapular + suprailliac) was calculated. Relative fat mass was derived from the formula of Durnin and Womersley (1974) of linear regression equations for the estimation of body density $\times 10^3$ (kg.m^{-3}) (Durnin and Womersley, 1974) in combination with Siri's equation for estimating body fat percentage (Siri, 1961):

$$\text{Body density (BD) (g.cm}^3\text{)} = c - [m \times (\Sigma 4 \text{ SF (mm)})]$$

Where:

C and M = standard age and sex-specific coefficients (Table 2.1).

Table 2.1; standard age and sex-specific coefficients (Durnin and Womersley, 1974).

		Age (yrs)	17-19	20-29	30-39	40-49	50 +
Male	c		1.1620	1.1631	1.1422	1.1620	1.1715
	m		0.0630	0.0632	0.0544	0.0700	0.0779
Female	c		1.1549	1.1599	1.1423	1.1333	1.1339
	m		0.0678	0.0717	0.0632	0.0612	0.0645

$\Sigma 4SF$ = sum of all four-site skinfolds (mm)

Once the density was calculated, the Siri equation was used to estimate body fat

Percentage:

$$\text{Body fat percentage (\%)} = [(4.95/d) - 4.5] \times 100$$

Where:

d= density, 4.95 and 4.5 = constants.

2.2 Expired air measurements

Oxygen uptake (VO_2) and carbon dioxide (CO_2) production was determined at rest, before and during exercise. Samples of expired air were collected into 100 or 150 L Douglas bags. While wearing a nose clip, subjects breathed through a mouthpiece fitted to a lightweight, large 2-way respiratory valve (2700 series, Hans Rudolph Inc. USA), which in turn was connected to a lightweight tube. The tubing was terminated at a two-way valve which opened and closed the Douglas bag. (All equipment was supplied by Cranlea & Co. Birmingham, England).

An aliquot of expired air (measured using a flow meter) was removed from each Douglas bag to determine the fraction of O_2 and CO_2 using a gas analyser (Servomex 4100, Servomex Group Ltd., East Sussex, England). The analyser was calibrated before each use with certified reference gases (BOC Ltd, Surrey, UK) and the reference gases were calibrated against a 'gold standard' reference gas to ensure consistency of results.

The remaining volume of expired air in each Douglas bag was measured by evacuation through a dry gas meter (Harvard apparatus, supplied by Cranlea & Co. Birmingham, England). The temperature of air in the Douglas bag was measured during evacuation using the same dry gas meter.

Barometric pressure was measured using a barometer and the measured expired gas volumes were corrected to standard temperature and pressure (STPD) for a dry gas using the universal gas equation. Inspired gas volumes were derived using the Haldane transformation (Consolazio et al., 1963) and O_2 uptake, CO_2 production, minute ventilation, respiratory exchange ratio and the ventilatory equivalent for oxygen were calculated. Rates of substrate utilization were calculated via indirect calorimetry using the equations described by Frayn (Frayn, 1983) and energy expenditure was determined by multiplying the mass of substrates used by their respective energy densities.

2.3 Monitoring of Heart Rate

The subject's heart rate was monitored continuously during exercise and the recovery period by short range telemetry (Polar S610i, Polar Electro, Finland).

2.4 Submaximal Exercise Test

Submaximal exercise test was performed to predict maximum oxygen consumption ($\text{VO}_{2\text{max}}$) for each subject prior to commencing main trials. The test was designed to exercise subjects through a range of intensities from moderate to vigorous but not maximum. The test consisted of four, continuous 5-min stages of walking on a treadmill to determine the relationship between gradient and oxygen consumption at self-selected walking speed of about 5 - 6 $\text{km}\cdot\text{h}^{-1}$ (see **Figure 2.1**). The first stage of the test was performed on a level treadmill and gradient was increased by 2.5-3.0% at the end of every stage depending on subject's heart rate response in the previous stage: if heart rate exceeded 100 beats per minute in the first stage, a 2.5% increment was used for subsequent stages. Each stage lasted five minutes with expired air being collected into Douglas bags during the last two minutes for the determination of VO_2 and VCO_2 by using the Douglas bag method. Five-minute stages were performed to ensure subjects were in steady state during expired air collection periods. Heart rate was recorded continuously during the test and the Borg scale was used to assess subject's perceived exertion simultaneously with the expired air collections at the end of every stage. The test was terminated if subject's heart rate reached 85% of his predicted maximum heart rate. At the end of the test, the oxygen uptake at each stage was plotted against the heart rate and gradient to estimate the gradient and speed necessary to elicit an intensity corresponding to 50% $\text{VO}_{2\text{max}}$ during the main trials.

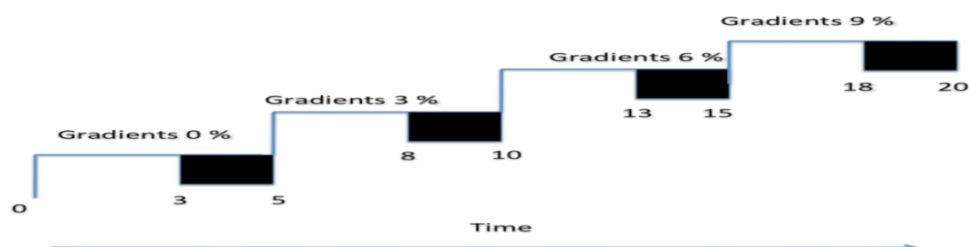


Figure 2.1. A schematic diagram of a 4-stage submaximal incremental test (black boxes represent expired air collection and heart rate measurements).

2.5 Test meals

Subjects were provided test meals as described in each of the three experimental chapters on completion of all fasting measurements. These were consumed within 10 minutes. The compositions of the meals were as follows:

2.5.1 Fat Loading Test (FLT) using high fat mixed meal (HFM)

The composition of the meal provided in **Chapter 4** and to a subset of participants in **Chapter 6** is shown in **Table 2.2**. The meal provided 1278 kcal of energy, of which 27% was carbohydrate, 65% was fat and the remaining 8% as protein. The ComplianTM formula was mixed thoroughly with whole milk and double cream to form a milkshake type drink. The croissant was lightly toasted, and spread with LurpackTM spreadable butter.

Table 2.2; Composition of the high fat mixed meal.

	Portion Size	Energy (Kcal)	Protein (g)	Total fat (g)	Total carbohydrate		
					Starch (g)	Sugar (g)	Total CHO (g)
Croissant (g)	100	430.0	9.2	25.3	34.8	5.8	40.6
Lurpack spreadable butter (g)	20	144.8	0.1	16.0	0.0	0.1	0.1
Complan (powder in g)	57	250.2	8.7	8.4	8.3	26.7	35.0
Whole milk (mls)	180	118.8	5.8	7.0	0.0	8.6	8.6
Double cream (mls)	75	333.8	1.3	35.6	0.0	2.0	2.0
Total	-	1277.6	25.1	92.4	43.1	43.2	86.3

2.5.2 Oral glucose Tolerance Test (OGTT)

This was provided to subjects in **chapter 5**. The drink prepared by dissolving 75 g of glucose in 275 ml of water and adding 25 ml of concentrated lemon juice. This provided 75 g of carbohydrate 0 g fat, 0 g protein and 300 kcal energy.

2.5.3 Oral fat Tolerance Test (OFTT)

This was provided to subjects in **Chapter 5**. This comprised 150 ml double cream, with 0.5 ml of sugar-free raspberry flavouring. This provided 2.4 g of carbohydrate 75 g fat, 2.3 g protein and 700 kcal energy (see **Table 2.3**).

Table 2.3; Composition of the OFTT.

Tesco™ double cream portions	
Portion Size (ml)	150
Energy (Kcal)	700
Protein (g)	2.3
Carbohydrate (g)	2.4
Fat (g)	75

2.5.4 Combined test (COMB)

This comprised the OGTT drink and the OFTT drinks described above and provided 77.4 g of carbohydrate 75 g fat, 2.3 g protein and 1000 kcal energy. This was provided to subjects in **Chapter 5** and a subset of participants in **Chapter 6**.

2.6 Blood sampling and processing

Venous blood was obtained via a cannula (Biovalve, 18G/1.2 mm, Vygon, France) placed in an antecubital vein. The cannula was kept patent by flushing with saline

solution (0.9% NaCl). Blood samples were collected directly in 10 ml tubes containing EDTA as an anticoagulant (**Chapter 4**) (BD Vacutainer Systems, Plymouth, UK) or in 6 ml SST™ serum tubes (**Chapters 5 and 6**) (BD Vacutainer Systems, Plymouth, UK). When EDTA tubes were used, they were placed immediately in ice, the cold temperatures (4 °C) inhibit glycolysis. The samples were centrifuged (GS-6KR, Beckman Instruments, Inc, California, US) within 15-30 min of collection for 15 minutes at 3000 rpm and 4 °C (WHO, 2006, Gambino et al., 2009). When serum tubes were used, samples were left in room temperature for 60 minutes and then centrifuged (GS-6KR, Beckman Instruments, Inc, California, US) for 15 minutes at 3000 rpm and 4 °C. EDTA plasma was pipetted into aliquots of 500 µl in 0.5 ml Apex tubes (0.5 ml, Alpha Laboratory Ltd, UK) and 2 x 350 µl in Apex tubes and frozen immediately at -70 °C, for subsequent analysis of insulin. Another 350 µl was placed in the fridge at 4 °C, for NEFA, glucose and lipid profile analysis within 24 hours. The remaining EDTA plasma (**Chapter 4**) or serum plasma (**Chapters 5 and 6**) was used for lipoprotein separation. This was started on the same day of blood collection.

Justification of using EDTA tube to measure glucose

The commonly used glycolysis inhibitors are unable to prevent short-term glycolysis. Glycolysis can be attenuated by inhibiting enolase with sodium fluoride (2.5 mg/mL of blood) or, less commonly, lithium iodoacetate (0.5 mg/mL of blood). These reagents can be used alone or, more commonly, with such anticoagulants as potassium oxalate, EDTA, citrate, or lithium heparin. Unfortunately, although fluoride helps to maintain long-term glucose stability, the rates of decline in the glucose concentration in the first hour after sample collection are virtually identical for tubes with and without fluoride, and glycolysis continues for up to 4 h in samples containing fluoride (Chan et al., 1989). After 4 h, the concentration of glucose in whole blood in the presence of fluoride remains stable for 72 h at room temperature (Chan et al., 1989) (leukocytosis will increase glycolysis even in the presence of fluoride if the leukocyte count is very high).

Few effective and practical methods are available for prompt stabilization of glucose in whole-blood samples. Loss of glucose can be minimized in two classic ways: 1) immediate separation of plasma from blood cells after blood collection [the glucose concentration is stable for 8 h at 25°C and 72 h at 4°C in separated, nonhemolyzed, sterile serum without fluoride (Burtis et al., 2012); and 2) placing the blood tube in an ice-water slurry immediately after blood collection and separating the plasma from the cells within 30 min (WHO, 2006, Gambino et al., 2009).

2.6.1 Plasma Analysis

Plasma glucose, insulin, TG, NEFA, total and HDL cholesterol and small dense LDL (Chapter 4, 5 and 6) and 3-hydroxybutyrate (Chapter 4) concentrations were analysed in the fasted state. In postprandial state TG, NEFA, glucose, insulin, sdLDL (Chapter 4, 5 and 6) and 3-hydroxybutyrate (Chapter 4) concentrations were analysed at all-time points. LDL cholesterol was calculated in the fasted state using the Friedewald equation (Friedewald et al., 1972), (Appendix C). All tests listed above were carried out by Mrs. Josephine Cooney, in the Vascular Biochemistry Department of McGregor building, University of Glasgow (Appendix C).

2.6.2 Lipoprotein separation

Principle

Flotation of lipoprotein particles through a gradient formed by layering solutions of decreasing density (generated using specific concentrations of NaCl, NaBr or KBr) above a sample of plasma adjusted to high density. (Lindgren et al., 1972).

Sequential preparation of chylomicrons ($S_f > 400$) by Ultracentrifugation

To separate the chylomicron fraction ($S_f > 400$), two ml of plasma were overlaid with 4 ml of 1.006 g.ml⁻¹ density solution in Ultra-Clear centrifuge tubes and spun at 10K rpm and 15°C for 30 min (Lindgren et al., 1972) using the Beckman L8-60M

Ultracentrifuge and Beckman 50.4 rotor (Beckman Instruments Inc., UK). The top 2 ml containing chylomicron particles ($d < 1.006 \text{ g.ml}^{-1}$) were removed (termed CM-1) and TG concentrations were measured using commercially available kits as described in **Appendix C**. This top fraction was used in the experiment mentioned in **chapter 3**. TG concentrations were also measured in the middle 1.5 ml fraction (CM-2) to verify complete collection of the CM fraction. The final chylomicron-TG concentration was calculated as the addition of these two fractions $[\text{CM-1} + (\text{CM-2} \times 1.5/2)]$ (**chapter 4** and **chapter 5**). The final 0.5 ml of the density solution overlay was discarded and the remaining 2 ml of chylomicron-free plasma was used for separation of VLDL₁ and VLDL₂. The CV for the chylomicron-TG separation using triplicate samples from the same participant was 4.9%.

Isolation of VLDL₁ (S_f 60-400), VLDL₂ (S_f 20-60), IDL (S_f 12-20) and LDL (S_f 0-12) Fraction by Swing-Out Ultracentrifugation

Two ml of the chylomicron-free plasma from the previous step was adjusted a density of $d \ 1.118 \text{ g.ml}^{-1}$ by the addition of 0.341 g NaCl. This was gently layered over a cushion of 0.5 ml $d \ 1.182 \text{ g.ml}^{-1}$ solution in an Ultra-Clear Beckman SW 50 ultracentrifugation tube (Beckman Instruments Inc., UK) which had been coated with polyvinyl alcohol (Holmquist, 1982); this allowed the solutions to be introduced down the side of the tubes smoothly without disturbing the formation of the gradient. A discontinuous gradient was formed by over-layering sequentially $d \ 1.0988 \text{ g.ml}^{-1}$ (1 ml), $d \ 1.0860 \text{ g.ml}^{-1}$ (1 ml), $d \ 1.0790 \text{ g.ml}^{-1}$ (2 ml), $d \ 1.0722 \text{ g.ml}^{-1}$ (2 ml), $d \ 1.0641 \text{ g.ml}^{-1}$ (2 ml) and finally $d \ 1.0588 \text{ g.ml}^{-1}$ (2 ml) (see **Table 2.4** below).

Table 2.4; Layers of density solutions

Density g.ml ⁻¹		Volume in ml	
-	1.182	0.5	bottom
Plasma	1.118	2	
1	1.0988	1	
2	1.0860	1	
3	1.0790	2	
4	1.0722	2	
5	1.0641	2	
6	1.0588	2	top

Centrifugation was carried out using a Beckman SW 40 rotor (Beckman Instruments Inc., UK) in Beckman L8-60M ultracentrifuge for 1.38 h at 39K rpm and 23 °C for separation of the VLDL₁ fraction. The rotor was decelerated without braking and by using a finely drawn glass Pasteur pipette the VLDL₁ fraction was removed in the top 1 ml. This volume was replaced by 1 ml of d 1.0588 g.ml⁻¹ and tubes were recapped and place back in the centrifuge overnight for separation of VLDL₂, for practical reasons, the times and speeds were calculated using g X minutes by applying a formula for conversion. The relationship between RPM and RCF is as follows:

$$RCF = 1.118 \times R \times (RPM \times 1000)^2$$

Where RPM is rotational speed (revolution per minute), RCF is relative centrifugal force, r is the radius of rotation measured in millimetres. R is constant here. Different speeds used for VLDL₂ separation are shown in **Table 2.5**.

At the end of the run, 0.5 ml of VLDL₂ fraction was removed using a finely drawn glass Pasteur pipette.

Tubes were recapped and placed back in the centrifuge for 2.35 h at 39K for separation of IDL. IDL was removed from the top of the tube as a 0.5 ml aliquot was withdrawn into Apex tubes and placed in the fridge for composition analysis. The tubes were recapped and placed into the rotor again and centrifuged overnight for separation of LDL, different speed and time could be applied (See **Table 2.5**). All lipoprotein subfractions have been stored in fridge at 4 °C to be analysed next day for composition.

Lipoprotein composition analysis

Lipoprotein fraction TG, phospholipid (PL), total cholesterol, free cholesterol (FC), cholesteryl ester (CE), (**Chapter 4 and 5**) were analysed by **Mrs. Josephine Cooney**, in the Vascular Biochemistry Department of McGregor building, University of Glasgow (**Appendix C**).

Table 2.5; Centrifugation conditions used for the separation of lipoprotein fractions.

chylomicron		VLDL ₁		VLDL ₂		IDL		LDL	
Time (h)	RPM	Time (h)	RPM	Time (h)	RPM	Time (h)	RPM	Time (h)	RPM
00.30	10K	1.31	39K	14.41	18.5K	2.35	39K	21.10	30K
Alternative conditions				12.03	21.1K	3.12	35K	17.30	33K
				17.31	17.5K	3.24	34K	16.29	34K
				18.08	17.2K	3.50	32K	18.36	32K
				14.52	19K			18.02	32.5K

2.6.3 Insulin Analysis

Principle

Mercodia Insulin ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to wells on microtitration plates. A washing step removes unbound enzyme labelled antibody. The bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically (Lequin, 2005).

Procedure

Plasma samples (25 μ l) were pipetted into the assay wells. 100 μ l of freshly prepared enzyme conjugate solution was then added to each well. Plates were incubated on a plate shaker for 1 hour at room temperature. During this incubation period, insulin in the samples reacted with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to plate wells. After incubation, the plates were washed and dried 5 times by an automatic washer to remove any unbound enzyme labelled antibody using the provided wash buffer solution. Bound conjugates which remained in the wells were detected by adding 200 μ l of 3,3',5,5'-tetramethylbenzidine (TMB). The plates were then incubated for 15 minutes at room temperature to allow reaction between substrate TMB and bound conjugates. After incubation, 50 μ l of the Stop solution containing 0.5 M sulphuric acid were added to each well to stop the reaction. A yellowish-tint colour developed according to the concentration of conjugate-substrate complex. The optical density of each well was read at 450 nm using a spectrophotometer. All samples were run in duplicate together with the standards ranging from 0 to 200 mU.l⁻¹. A standard curve was obtained using cubic spine regression. The concentration of insulin in the samples was then determined by comparing the optical density of the samples to that of the standard curve for each respective plate. All reagents and samples were brought to room temperature before use. Coefficients of variation between assays were <5%.

2.6.4 Insulin resistance estimated the homeostasis model

The homeostasis model assessment-estimated insulin resistance (HOMA-IR), developed by Matthews et al. (Matthews et al., 1985) has been widely used for the estimation of insulin resistance. Due to the simplicity of its determination and calculation, HOMA-IR has been the most frequently employed technique both in clinical practice and in epidemiological studies. HOMA-IR was calculated in **Chapter 6** using this formula (fasting plasma insulin in mU.l^{-1} X fasting plasma glucose in mmol.l^{-1})/22.5 (Wallace et al., 2004). Low HOMA-IR values indicate high insulin sensitivity, whereas high HOMA-IR values indicate low insulin sensitivity (insulin resistance)(Matthews et al., 1985).

2.7 Lowry assay

Principle

Protein is measured by the addition of Biuret Reagent and Folin-Ciocalteu reagent, which produces a colour change to blue. The colour intensity, which is proportional to the concentration of the protein present, is measured by optical density (OD) at 750 nm using a spectrophotometer. The protein concentration is calculated using a standard curve of known concentrations. The following method is a modification from the original. All volumes have been halved (Lowry et al., 1951).

Procedure

Total protein was measured by adding 1 ml of Biuret Reagent and Folin-Ciocalteu reagent, [100 ml of 2% Na_2CO_3 in 0.1 M NaOH (w/v), 1 ml of 2% NaK Tartrate (w/v), 1 ml of 1% CuSO_4 (w/v), and 1 ml of 10% (w/v) sodium dodecyl sulphate (SDS)] to a total volume of 200 μl of sample (100 μl VLDL₁ + 100 μl of distilled water or 50 μl VLDL₂ + 150 μl distilled water). One hundred microlitre of 1:1 Folin-Ciocalteu reagent (Sigma-Aldrich Company Ltd., Irvine, Scotland) was then added and mixed immediately. After incubation at room temperature for 30-60 min, the developed colour intensity was measured at an optical density of 750 nm using a Beckman DU 70 Spectrophotometer. The protein concentration was calculated using a

standard curve of known concentrations. Inter-assay precision was checked using 2 levels (100 μl and 200 μl) of human and bovine quality control (QC) materials (Lowry et al., 1951). The coefficients of variation (CVs) for the low QC were (human: 1.8%, bovine: 1.3%) and high QC were (human: 1.0%, bovine: 1.4%).

2.8 LPL assay

Principle

Triglycerides are hydrolysed by lipoproteinlipase to produce glycerol and free fatty acids. In order to apply this reaction a medium should be prepared to run the experiment.

Procedure

Tris-HCL buffer Preparation

Tris-HCl buffer was prepared by mixing; 24.22 g.l^{-1} Tris-HCl (200 mmol.l^{-1}) (Sigma-Co NO. T3253), 366.24 mg.l^{-1} of CaCl_2 (3.3 mmol.l^{-1}) (Sigma-Co NO. C4901), 7597.59 mg.l^{-1} of NaCl (130 mmol.l^{-1}) (VWR C-S9888) and 66.7 mg.l^{-1} of sodium-heparin (0.010 mmol.l^{-1}) (Sigma-Co NO. H3393) to stabilize LPL and this inhibits the release of the attached LPL from VLDL, in a pH level of 8.2. To reach the required pH level HCl, or NaOH were used (Zambon et al., 1996, Saheki et al., 1991). Different concentrations of LPL were prepared by diluting the stock solution in Tris-HCl buffer.

2.9 Statistical analysis

2.9.1 Power calculations

An *a priori* power calculation, on the basis of our data for intra-subject reproducibility of postprandial TG responses in men (between-day coefficient of variation 10.1%) indicated that 10 participants would enable detection exercise-induced changes of ~10 % in the TG response with 80 % power (Gill et al., 2005). Significance was accepted at the $p < 0.05$ level. Data are presented as means \pm SEM unless otherwise stated.

2.9.2 Calculation of summary postprandial responses

Time-averaged postprandial concentrations, calculated as the trapezium rule-derived areas under concentration versus time curve, divided by the duration of the postprandial observation period, were used as summary measures of the postprandial responses.

2.9.3 Normality of data

The normality tests are supplementary to the graphical assessment of normality (Elliott and Woodward, 2007). There are many tests to assist normality (Elhan and Tuccar, 2006, Peat and Barton, 2008). In **chapter 4,5 and 6** normality of data was tested using Anderson-Darling test (Elhan and Tuccar, 2006).

If a variable did not fit a normal distribution or had greatly different standard deviations in different groups, data transformation was applied (Tu and Xia, 2008, Chou et al., 1998). Where data did not approximate a normal distribution, these were log-transformed prior to analysis and data are expressed as geometric means with 95% confidence intervals (95% CI).

2.9.4 Significant testing

Data was analysed using Statistica (version 10, StatSoft Inc.) and Minitab (version 17, Minitab Ltd).

Comparisons between interventions were made using paired *t*-tests, where a single pair of means was compared. The validity the test based on normality of the data, the sample is a simple random sample from its population and data is continuous of the data.

When comparisons were made between groups or across multiple time-points as in **Chapter 4** or in different trials as in **chapter 5** and **6** a two-way repeated measures ANOVA, with *post hoc* Fisher tests, were used. The validity of the test based on, homogeneity of variance, independency of the sample and normality of the data.

2.9.5 Cohen's *d*, effect size

In comparing different intervals, it is useful to express the size of the effect in standardized way. One common way is to report Cohen's *d* effect size, which is relating the effect size to the variance. Statistical significance depends on the sample size and the precision of the data. When a large set of data analysed, very small effects may reach statistical significance. Therefore, it is useful to determine effect size to describe, if effects have a relevant magnitude and to describe the strength of a phenomenon. The most popular effect size measure is Cohen's *d* (Cohen, 2013). Effect size is a standard measure that can be calculated from any number of statistical outputs (Cohen, 2013).

Cohen's '*d*', expresses the mean difference between two groups in standard deviation units. Typically, this is reported as Cohen's *d*, or simply referred to as "*d*." Though the values calculated for effect size are generally low, they share the same range as standard deviation (-3.0 to 3.0), so can be quite large. Interpretation depends on the research question. The meaning of effect size varies by context, but the standard interpretation offered by Cohen (1988) is:

0.8 = large (8/10 of a standard deviation unit)

0.5 = moderate (1/2 of a standard deviation)

0.2 = small (1/5 of a standard deviation)

Reporting effect size helps to: understand the importance of an effect (Kirk, 1996), comparing the effect size within or between studies, and secondary analysis such as power calculations or meta-analysis (Reiser and Faraggi, 1999).

The effect size of the interventions was calculated in **Chapter 4 and 5**. The effect size was calculated differently in **chapter 6**. The mean difference was calculated for each individual then divided by the SD of both groups (Cohen, 2013).

3 Development of a Method to Determine the Susceptibility of Triglyceride-Rich Lipoproteins for Hydrolysis

Lipoprotein lipase is the major enzyme which hydrolyses triglycerides (TG) present in the circulation to release free fatty acids (FFA). It is the rate-limiting enzyme controlling plasma TG clearance. Hydrolysis of TG is essential for energy storage and utilization (Goldberg and Merkel, 2001, Wang and Eckel, 2009). Elevated postprandial TG concentrations are independently associated with risk of cardiovascular events and atherosclerotic disease process (Chapman et al., 2011, Goldberg IJ, 2011, Bansal et al., 2007). Considerable attention over the last decades has been focused on the effect of exercise on postprandial metabolism and its potential role in the reduction of fasted and postprandial TG. Exercise is considered an effective method to lower postprandial TG concentrations by about 20-25% (Farah and Gill, 2012, Gill et al., 2004a). The mechanism(s) by which exercise induces triglyceride lowering is currently not clear. It has been hypothesised that exercise may cause compositional changes to the VLDL₁ particles, which are the major determinants of plasma TG concentrations (Austin, 1990, Patsch et al., 1992, Deckelbaum et al., 1984), thereby increasing their clearance by LPL (Al-Shayji et al., 2012). Therefore, in order to test this hypothesis, it was essential to develop a method to test the susceptibility of chylomicron and VLDL₁ to TG hydrolysis in standardised assay (described in Chapter 4 as “affinity of TRL for LPL”). In this chapter, the development and optimisation of this assay, based a method previously described by (van Barlingen et al., 1996).

3.1 Elements of standardised lipolysis assay

The ultimate aim of this study is to develop a method to determine how exercise influences the susceptibility of TRL to lipolysis. This requires development of a standardised assay to determine the rate of TG hydrolysis when the concentrations of substrate TRL and enzyme are fixed.

To generate an appropriate working method, a number of factors need to be considered (see **Figure 3.1**). These include:

- 1) Determining of optimal indicator of lipolysis (i.e., TG decrease or NEFA / glycerol rise).
- 2) Standardizing the concentration of lipoprotein particles.
- 3) Determining optimal concentration of LPL.
- 4) Determining the optimal assay conditions including:
 - a) Duration of the assay.
 - b) Optimizing the inhibitor of the reaction.
 - c) Albumin interference.

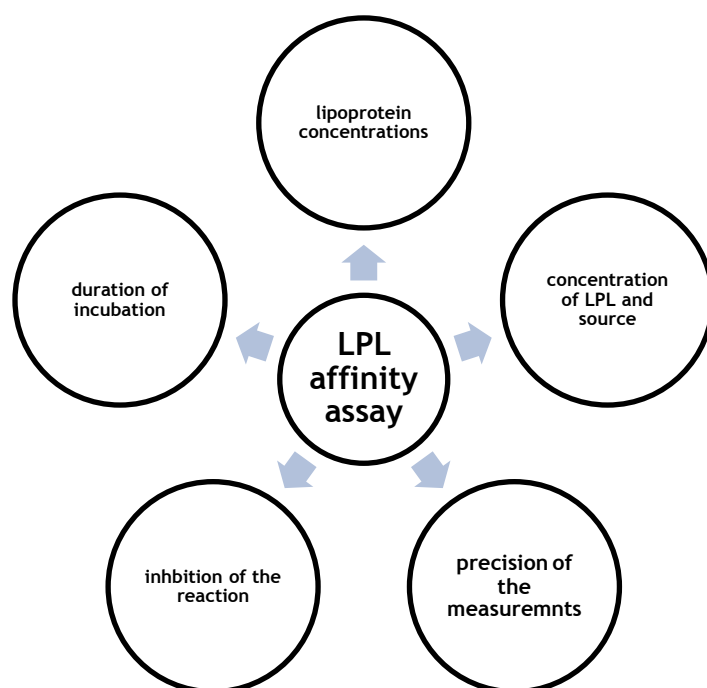


Figure 3.1. Conditions that been addressed in lipolysis assay include, standardizing TRL, LPL concentration and optimal assay conditions.

3.1.1 Choice of indicator of rate of lipolysis

The principle based on the hydrolysis of TG by the action of LPL producing three NEFA and glycerol (Fahy et al., 2009):



There is no gold standard or universal method for LPL measurement. To measure LPL activity in a closed system, any of the following products can be assessed in principle:

- (a) the rate of degradation of the TG
- (b) the rate of production of fatty acids or glycerol (Smeltzer et al., 1992) (See Figure 3.2).

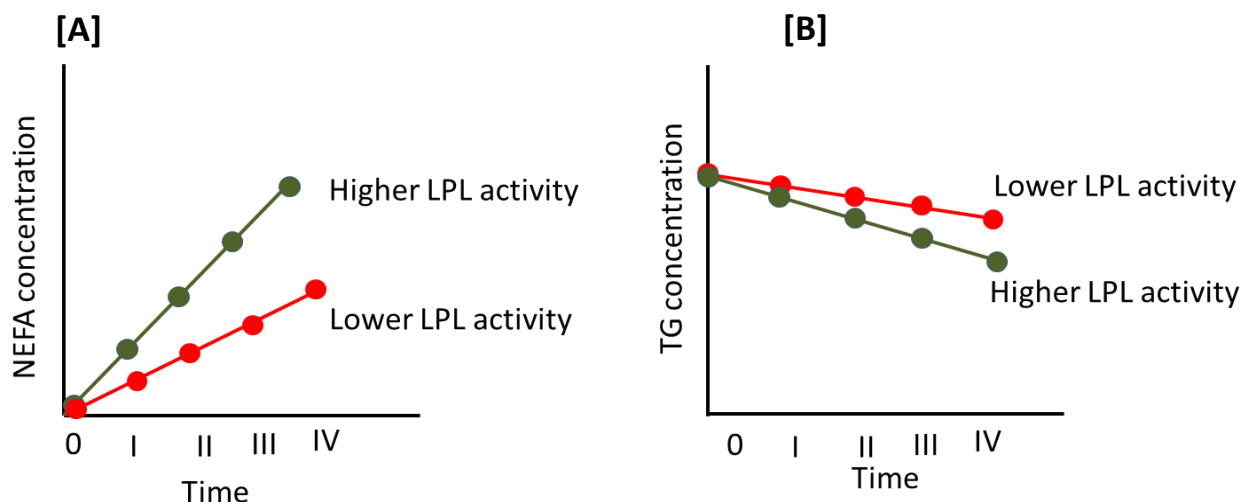


Figure 3.2. The products of TRL hydrolysis, panel [A] shows the rise in NEFA, panel [B] shows the degradation of TG; both can be used as a parameter of the reaction. NEFA concentration will rise as triple as TG will fall. The higher TG will breakdown the higher NEFA will be released. The rate of the reaction will increase when a higher activity enzyme is used.

Thus, by incubating TRL with LPL for over a range of time intervals, it is possible to calculate the rate of LPL activity.

The assay was initiated in a medium contains Tris-HCl with a pH of 8.2. The initial volume of the reaction was; 250 µl lipoprotein incubated with 125 µl of albumin and 125 µl of Tris-HCl buffer at 37 °C for 10 min to climate the tube. A 125 µl of LPL were added in each tube except the control, and incubated for different time points 0, 5, 10, 15, 20, 30, 60, and 90 mins. Each tube was quenched by 500 µl of pre-cooled NaCl (1 M) (Table 3.1). Then assay the products of the reaction (TG, glycerol and NEFA) to assess TG hydrolysis rate.

Table 3.1; initial assay mixture.

Time (min)										
All in µl	Control									
	0	0	0	5	10	15	20	30	60	90
Tris-HCl	125	125	125	125	125	125	125	125	125	125
1.006 g.ml ⁻¹	250	0	0	0	0	0	0	0	0	0
LPL	0-	250	0	125	125	125	125	125	125	125
Albumin	125	125	125	125	125	125	125	125	125	125
lipoprotein	0	0	250	250	250	250	250	250	250	250
NaCl	500	500	500	500	500	500	500	500	500	500
Total volume	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

3.1.2 Triglyceride measurements

Method

Initially, two hydrolysis assays were carried out using constant concentration of VLDL₁ (0.10 mmol.l⁻¹) as substrate 100 units of LPL during 30 minutes interval. Followed by another assay, using higher doses of VLDL 0.25 mmol.l⁻¹ of and 400 units of LPL during 30 minutes interval.

Result

As shown in **Figure 3.3, Panel [A] and [B]**, the rate of NEFA was increasing with time. **Figure 3.3, Panel [B]**, shows that with increasing the TRL and LPL concentrations, more NEFA were released. However, TG concentration remained constant in both trials.

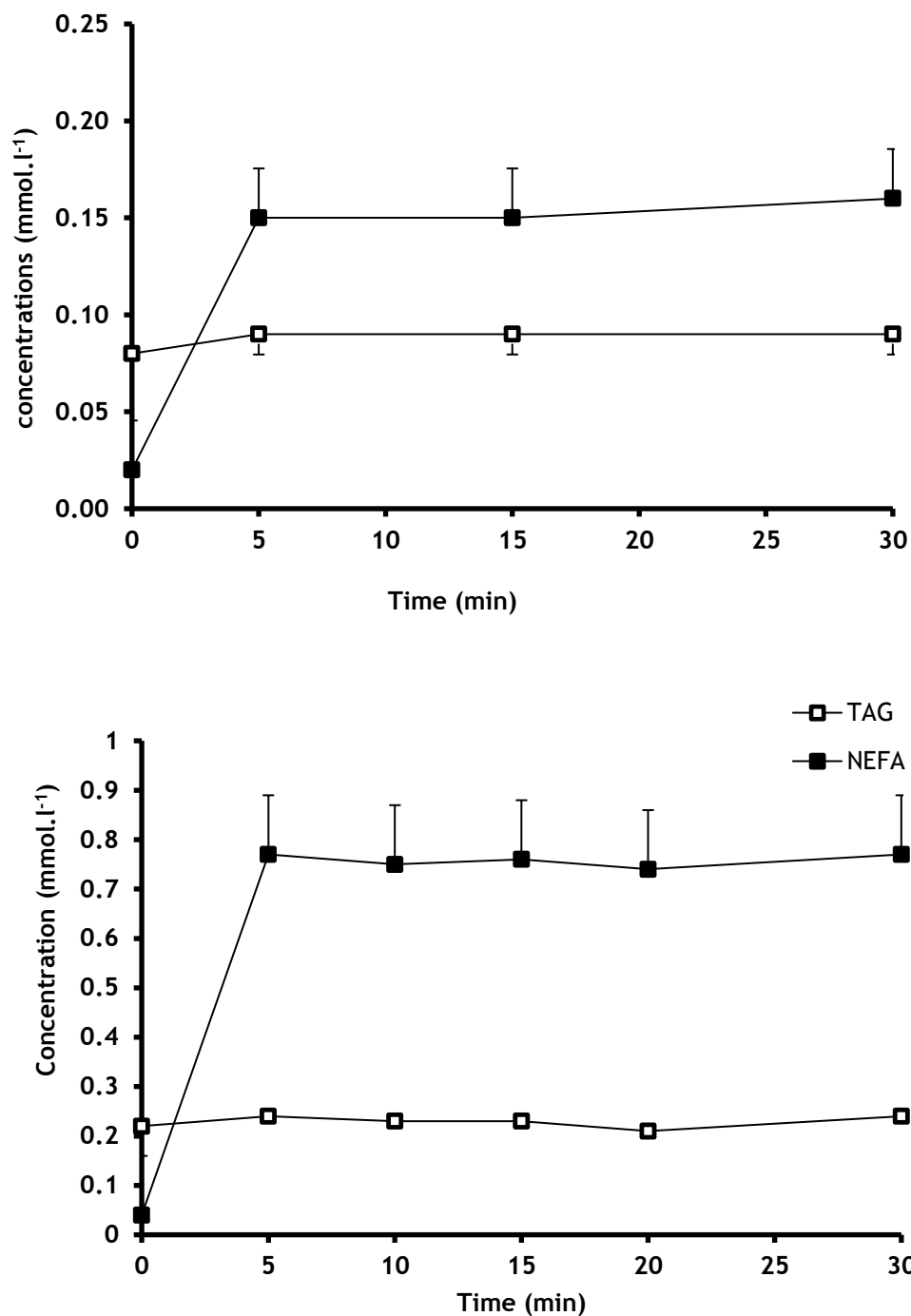
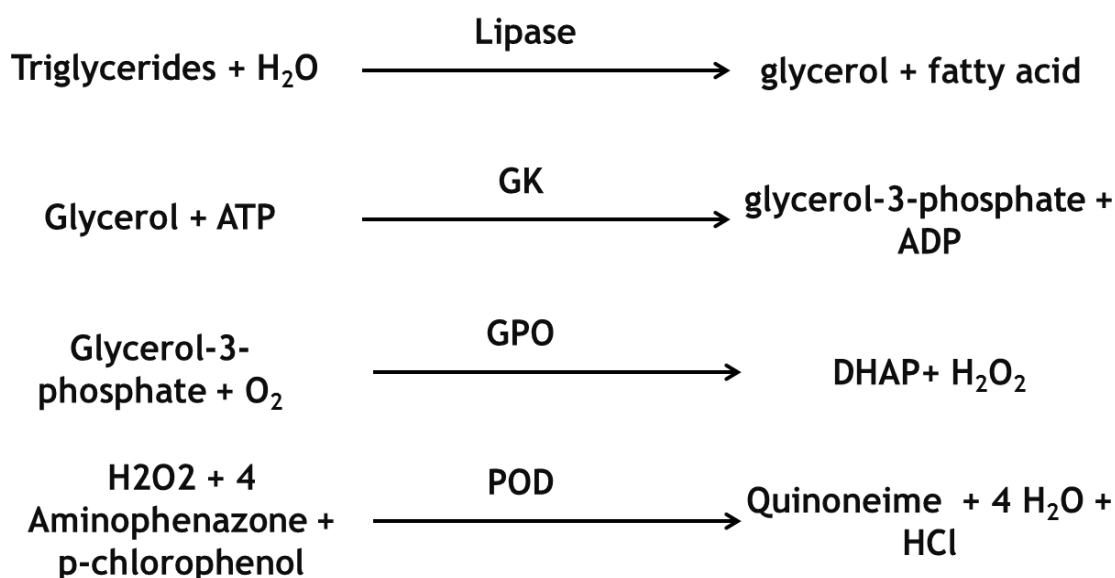


Figure 3.3. Release of NEFA and degradation of TG from VLDL₁ during 30-minutes observation, Panel [A] shows the release of NEFA and degradation of TG from (0.10 mmol.l⁻¹) of VLDL₁ and 100 units of LPL, Panel [B] shows release of NEFA and degradation of TG from (0.25 mmol.l⁻¹) VLDL₁ and 400 units of LPL.

TG concentration was constant because the assay that was used to detect TG in ILab-600 based on hydrolysing TG into glycerol and this result in measuring all the glycerol in the mixture. The principle of the reaction is as the following:

Principle:

The principle for this reaction is based on lipase hydrolysis of TG to glycerol followed by oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide produced then reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase to form a red dyestuff which was measured spectrophotometrically using the IL600 analyser at 550 nm. The equation is shown below:



(GK: Glycerol kinase; GPO: Glycerol phosphate oxidase; POD: Peroxidase; DHAP: dihydroxyacetate phosphate). The CV for the assay was 3.8%.

Moreover, it has been reported that a single action of LPL would not release glycerol, it would release monoglyceride and diglycerides (Gilham and Lehner, 2005). Therefore, glycerol was measured later on and the TG concentrations were corrected by subtracting glycerol.

3.1.3 Glycerol measurements

Method

Hydrolysis assay was carried out using different concentrations of VLDL₁ and constant amount of LPL. VLDL₁ at a concentration of 0.5 mmol.l⁻¹ was incubated with 0.1 unit of LPL.

Result

As shown in **Figure 3.4**, TG concentrations did not decrease during the 30 minutes interval; however, the glycerol and NEFA concentrations were not increasing as desired. Moreover, it has been observed that, glycerol measurement appeared not to be reproducible. Therefore, we measured the inter-assay CV of free glycerol kit used on the ILab-600.

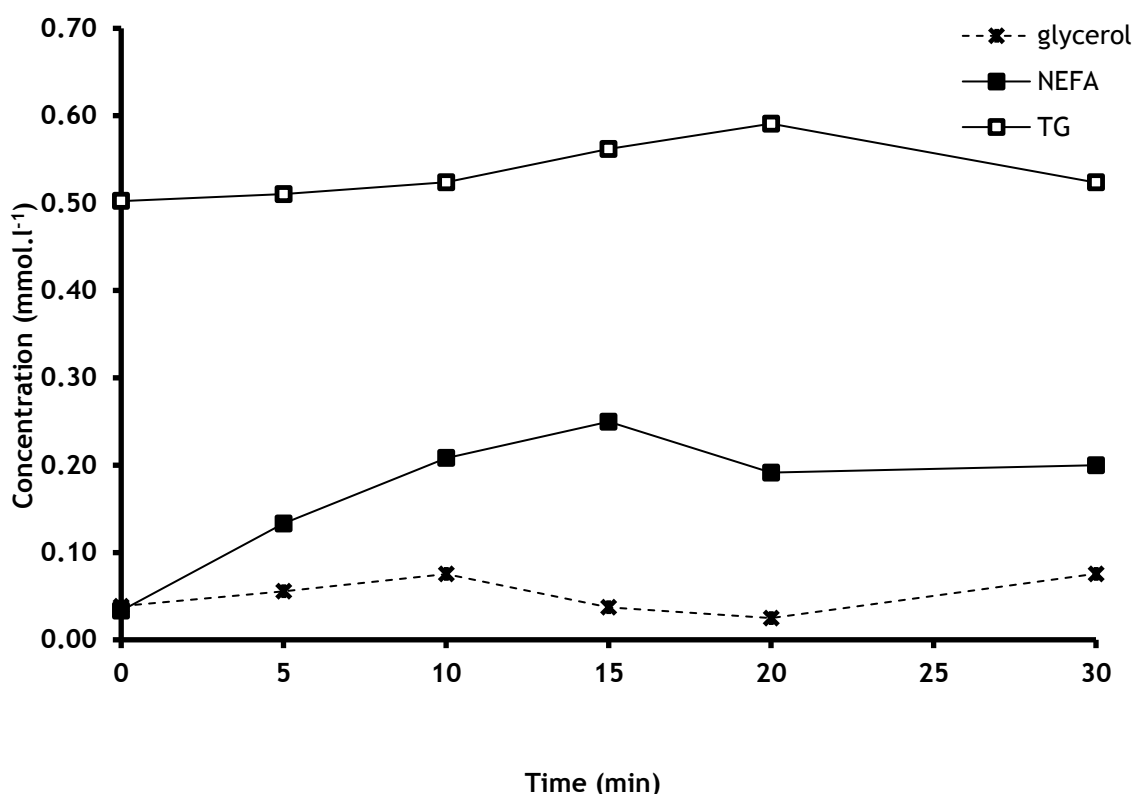


Figure 3.4. Release of NEFA and glycerol and degradation of TG from 0.5 mmol.l⁻¹ VLDL₁ and 0.1 units of LPL concentrations during 30-minutes observation.

3.1.4 I-Lab reproducibility

The glycerol measurement was fluctuating, thus we tested the reproducibility of ILab-600 by running 10 times repeated measure using aliquots of same participants. The Initial results from the measurement of glycerol concentrations using the automated ILab-600 analyser in whole plasma, chylomicrons, VLDL₁, and VLDL₂, revealed are shown in **Table 3.2**. It was clear that the CV for glycerol at the required concentrations was very high, an order of magnitude higher than that for TG or NEFA, but CVs for TG and NEFA were also higher than expected. This prompted servicing of the analyser, where a new lamp and pump were fitted, with a system wide clean of the analyser. Additionally, measurements for NEFA, TG, and glycerol were being taken simultaneously within each sample, however further examination found that the reagents for ensuring TG and glycerol were having a carryover of NEFA. Therefore, NEFA, glycerol, and TG were measured separately and in that order thereafter. However, based on these data, it was felt that the rise in NEFA concentration provided the best signal for LPL hydrolysis.

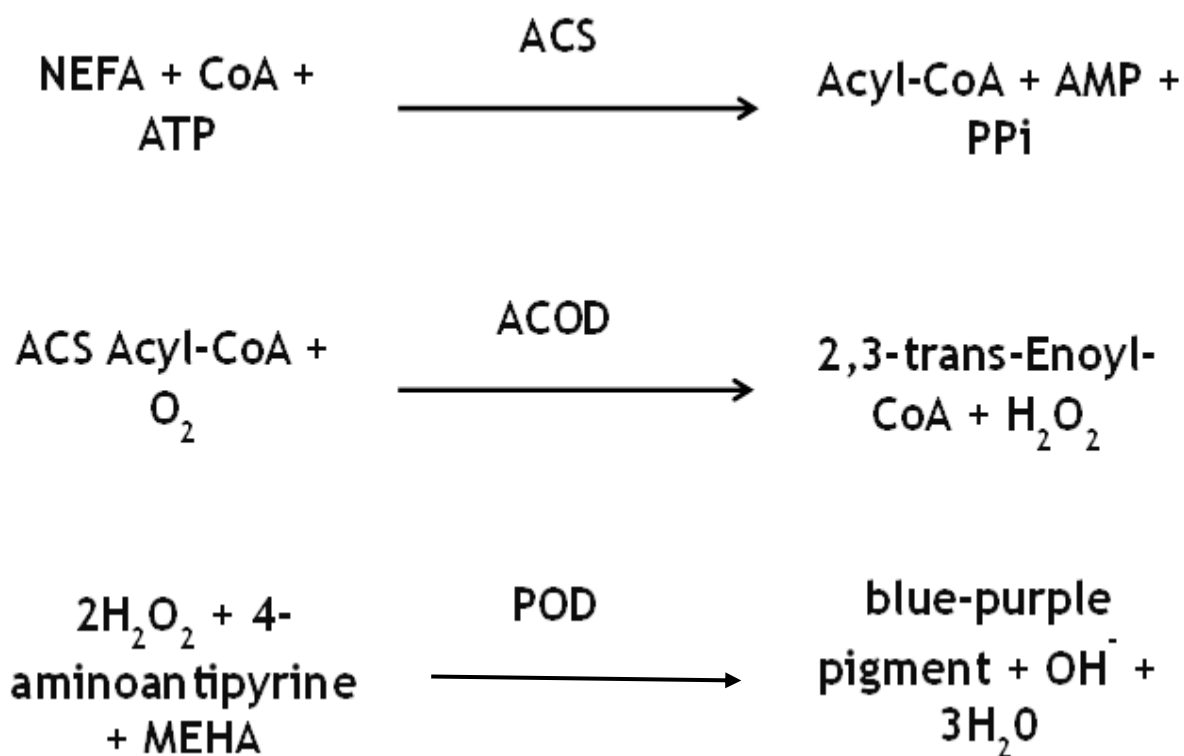
Table 3.2 ; reducibility of glycerol measurements in ILab-600.

	Glycerol	CV%	TG	CV%	NEFA	CV%
Plasma	0.06 ± 0.01	1.6 %	1.42 ± 0.01	0.81%	0.39 ± 0.09	1.2%
1:5 diluted plasma	0.02 ± 0.001	75%	0.33 ± <0.01	1.2%	0.14 ± 0.07	5%
1:10 diluted Plasma	0.009 ± 0.008	85%	0.20 ± <0.01	5%	0.09 ± 0.02	10%
Chylomicrons	0.02 ± 0.004	17%	0.14 ± 0.01	3.5%	0.16 ± <0.01	16%

3.1.5 Albumin Interference

As it has been reported above, the NEFA values were at the lower borderline of ILab detection limit. This has been reported previously. A number of studies have shown that serum albumin caused a decrease in NEFA detection when using the same reaction (Duncombe, 1963, Duncombe, 1964, Matsubara et al., 1983). The albumin complexes with NEFA preventing it from binding to the active site of ACS, thus preventing it from joining with CoA to form Acyl-CoA, and hindering the detection reaction (Matsubara et al., 1983).

The enzymatic reaction used to quantify the concentration of NEFA by the Waco kit is based on the Dole method (Dole, 1956), which involves the covalent binding of NEFA with Coenzyme A (CoA) to form Acyl-CoA. This reaction is catalysed by the enzyme Acyl-CoA synthetase (ACS).



ATP = adenosine triphosphate; ACS = acyl-CoA synthetase; AMP = adenosine monophosphate; PPi = pyrophosphate; ACOD = acyl-CoA oxidase; MEHA = 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline; POD = peroxidase

Therefore, we ran an assay using different concentrations of albumin to investigate previous assays 5% of albumin was added to each sample to sequester excess-background NEFA, which in high concentrations has been shown to inhibit LPL activity (Bengtsson and Olivecrona, 1980). Therefore, we opted to compare the concentration of NEFA detected in an assay without, and with 2%, and 5% albumin.

Result

Figure 3.5, Panel [A], shows that after a 30 minute of incubation of chylomicron with 0.1 unit of LPL and without albumin, there was 14% more NEFA released than with albumin. For VLDL₁ when incubated without albumin, there was an over 2-fold increase in detected NEFA concentration over its albumin containing counterpart (**Figure 3.5, panel [B]**). Without albumin, there was a steady increase in NEFA concentration over the 20 minute period, however, with albumin the NEFA concentration appeared to be levelling off after 10 minutes, but more data points are needed for both conditions to tell conclusively. Based on these data, albumin was removed from the LPL assay and the volume deficit has been replaced with Tris-HCl buffer. A final step of a sonication of each sample for 2 minutes before analysis to remove interference from bubbles was also included.

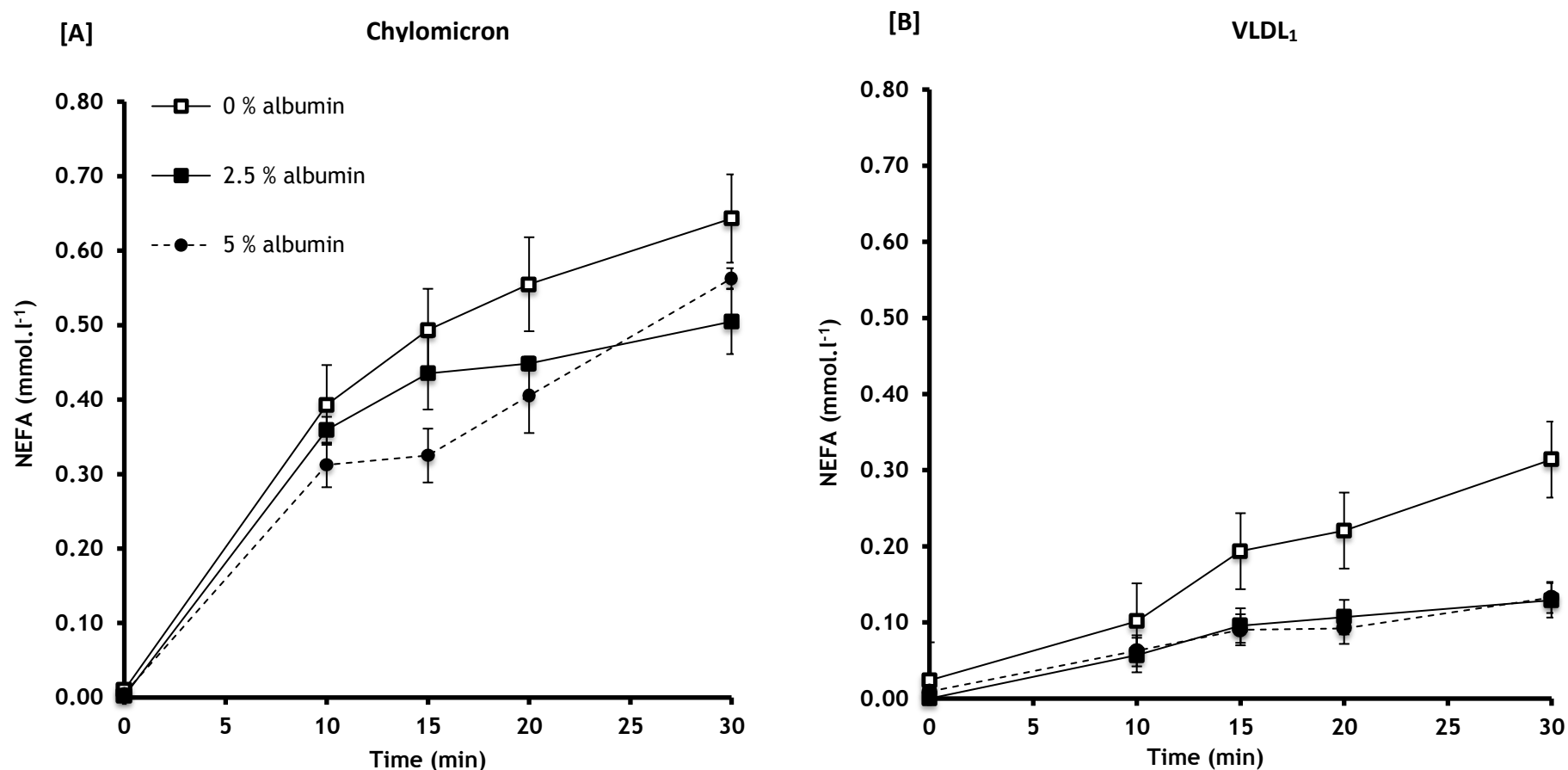


Figure 3.5. The effect of adding albumin to the hydrolysis assay during 30m interval. Panel [A] shows the release of NEFA from chylomicron when different albumin concentrations were added to the assay mixture, panel [B], shows the degradation of TG in chylomicron when different albumin concentrations were added to the assay mixture, panel [C], shows the release of NEFA from VLDL₁ when different albumin concentrations were added to the assay mixture, panel [D], shows the degradation of TG in VLDL₁ when different albumin concentrations were added to the assay mixture.

3.1.6 Conclusion

The increase in NEFA concentration, rather than the degradation of TG and rise of glycerol as final products of the reaction was used as an indicator of TRL hydrolysis. In addition, it was decided TG and NEFA were measured separately in the ILab-600 to avoid any interference that might affect the readings. The removal of albumin helped in better detection of NEFA. These modifications allowed us to reach a CV% of <1% for NEFA at concentration of 0.18 mmol.l⁻¹ and 2.1% at concentration of 0.15 mmol.l⁻¹ for TG when measured in ILab-600.

3.2 Standardisation of TRL concentration

3.2.1 Aim

To accurately and precisely determine TRL susceptibility to TG hydrolysis it is necessary to ensure that TRL concentration in the assay is standardised. The aim here was to optimise a process to concentrate TRL particles to enable easy dilution to a single standardised concentration for all TRL (chylomicrons, VLDL₁ and VLDL₂) in all individuals under all conditions (fasted and postprandial, control and exercise). **This would also enable the use of a concentration high enough to be read accurately and precisely by the ILab-600.**

3.2.2 Method

Subjects were fasted for >12 h before a high fat meal was given (see **Section 2.5.1**). Fifty ml of fasting and postprandial (4 hours after meal ingestion) blood was collected from rested subjects in EDTA tubes and was placed immediately on ice. Plasma was separated within 15 minutes of collections as previously mentioned in **Section 2.6**.

Concentration of chylomicrons

Chylomicron particles were separated from postprandial plasma samples as described in **section 2.6.2**. Chylomicrons were then concentrated in this sample using washed 10000 MWCO Centrifugal Filter Units (Fisher scientific, UK). The Centrifugal Filter Units were washed with distilled water 4-5 times at 2400 rpm for 13-23 minutes to remove any traces of glycerol.

Each subfraction was then centrifuged at 3000 rpm for 90 minutes until a constant volume was produced, giving a 2-10X concentrated sample. Concentrated chylomicron was stored at 4 °C (**Figure 3.6**).

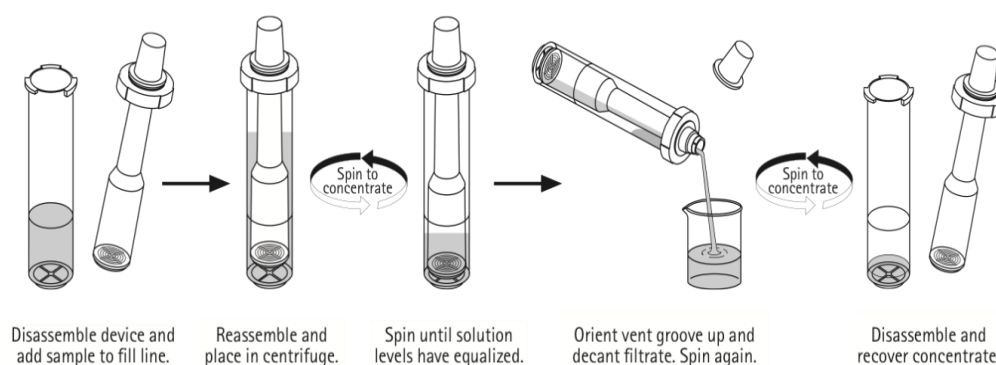


Figure 3.6. Chylomicron were concentrated using filtered tubes at 3000 rpm at 4°C for 90 minutes.

VLDL particles

It was thought that VLDL₁ and VLDL₂ particles may not be stable once they were separated from plasma; therefore, we assessed the stability for both particles, by, measuring concentrations of TG in samples stored for 0, 24 and 48 hours. Both subfractions were stable for 48 h in the fridge at 4 °C.

After the removal of chylomicron particles as detailed in **Section 2.6.2**, plasma was adjusted to a density of 1.25 g.ml⁻¹ by the addition of 0.3517 g.ml⁻¹ potassium bromide (KBr; BDH: 101954F), 15 ml of 1.25 g.ml⁻¹ density solution (1.006 g.ml⁻¹ density Solution + 0.3517 g.ml⁻¹ of KBr) was overlaid on 10 ml 1.25 g.ml⁻¹ plasma in Ultra-Clear centrifuge tubes (**Figure 3.7**), which was then ultracentrifuged at 39 k rpm for 46 hours at 15°C using the Beckman L8-60M Ultracentrifuge and Ti 70 fixed angle rotor (Beckman Instruments Inc., 337922, UK). The samples were left for 5-10 minutes after centrifugation to settle, before the top 2 ml was aspirated and reserved for VLDL separation as it mentioned in **section 2.6.2**. This resulted in a 5-fold concentration of TRL. The ultracentrifugation process to separate VLDL leads to a further doubling of the VLDL₁ concentration (as 2 ml of the initial solution is collected in 1 ml) and 4-fold increase in VLDL₂ concentration (as the fraction is collected in 0.5 ml). Thus this process leads to a 10-fold increase in concentration of VLDL₁ and 20-fold increase in concentration of VLDL₂.

3 *Development of a Method to Determine the susceptibility of Triglyceride-Rich Lipoproteins for hydrolysis*



Figure 3.7. 70 Ti rotor and tubes, used to concentrate VLDL particles.

3.2.3 Result

After concentration of TRL, typical concentrations achieved were 1.0 mmol.l^{-1} for chylomicrons, 5.0 mmol.l^{-1} for VLDL_1 and 3.0 mmol.l^{-1} for VLDL_2 . Thus, to ensure that a reproducible concentration of TRL could be achieved in all participants in all conditions (including following exercise, where concentrations could be reduced by up to 40%), it was decided to standardise TRL concentrations in the assay at 0.6 mmol.l^{-1} .

3.3 Optimising lipase amount

3.3.1 Aim

To determine suitable concentration of LPL to hydrolyse TRL to release NEFA. This includes testing the concentration of LPL and the volume of TRL in the assay. Further questions were addressed included whether the enzyme needed an activator and the incubation period. A previously described assay was used as the starting point (van Barlingen et al., 1996).

3.3.2 Methods

Serial assays were conducted using different LPL concentrations from *Burkholderia* sp. The above separated VLDL₁ particles were incubated with LPL in Tris-HCl buffer and then quenched with different NaCl concentrations. A number of adjustments were applied to reach the optimal assay conditions as following;

LPL concentrations and reaction duration

A range of LPL concentrations were used (100, 200, 400 and 800 units) for a range of incubation periods with TRL up to 90 minutes.

As shown in **Figure 3.8** the release of NEFA increased by increasing LPL concentrations. Also, it observed that most of the reaction occurred in the first 5-10 minutes and saturation was generally seen within the first 10 minutes.

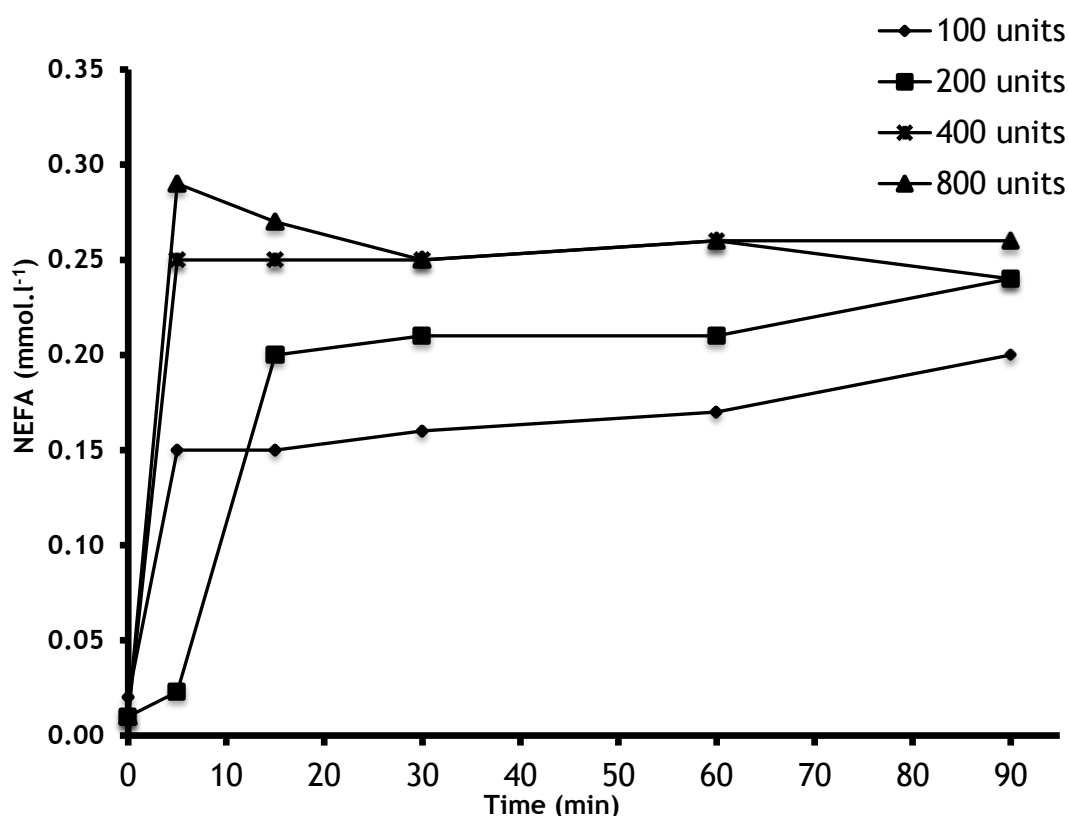


Figure 3.8. Values for NEFA release over 90-minutes in LPL-affinity assay in VLDL₁ using different LPL concentrations; 100, 200, 400 and 800 units.

The reactions were fast and reached the saturation point quickly. In addition, concentrations of NEFA were low, which providing challenges for accurate and precise measurement using the iLab 600 analyser. This was not optimal; to measure the affinity of LPL after exercise a slower reaction was needed to be able to observe the change. Thus much lower concentrations of LPL were used in a second set of assays as described below. In addition, the volume of the reaction mixture was reduced by reducing the volume of buffer to increase final NEFA concentrations.

3.3.3 Final enzyme concentrations

In order to achieve a slower action of the reaction, a decision was made to reduce the concentrations of LPL substantially and to try LPL from different sources. Thus, experiments using 0.1 unit of LPL from *Pseudomonas* sp. and *Burkholderia* sp. were performed. In addition, the volume of the mixture was adjusted from

500 μ l to 140 μ l as seen in Table 3.3, to increase the final concentration of NEFA in the reaction mixture VLDL₁ was incubated with 0.1 units of LPL from both sources (Figure 3.9).

With 0.1 units of LPL from *Pseudomonas* sp. and a reaction mixture volume of 140 μ l, the rate of NEFA released over the incubation period was linear until 20 minutes with NEFA concentrations within a range which enabled accurate and precise detection by the ILab-600 analyser. In contrast NEFA release using LPL from *Burkholderia* sp. was negligible over the incubation period (Figure 3.9).

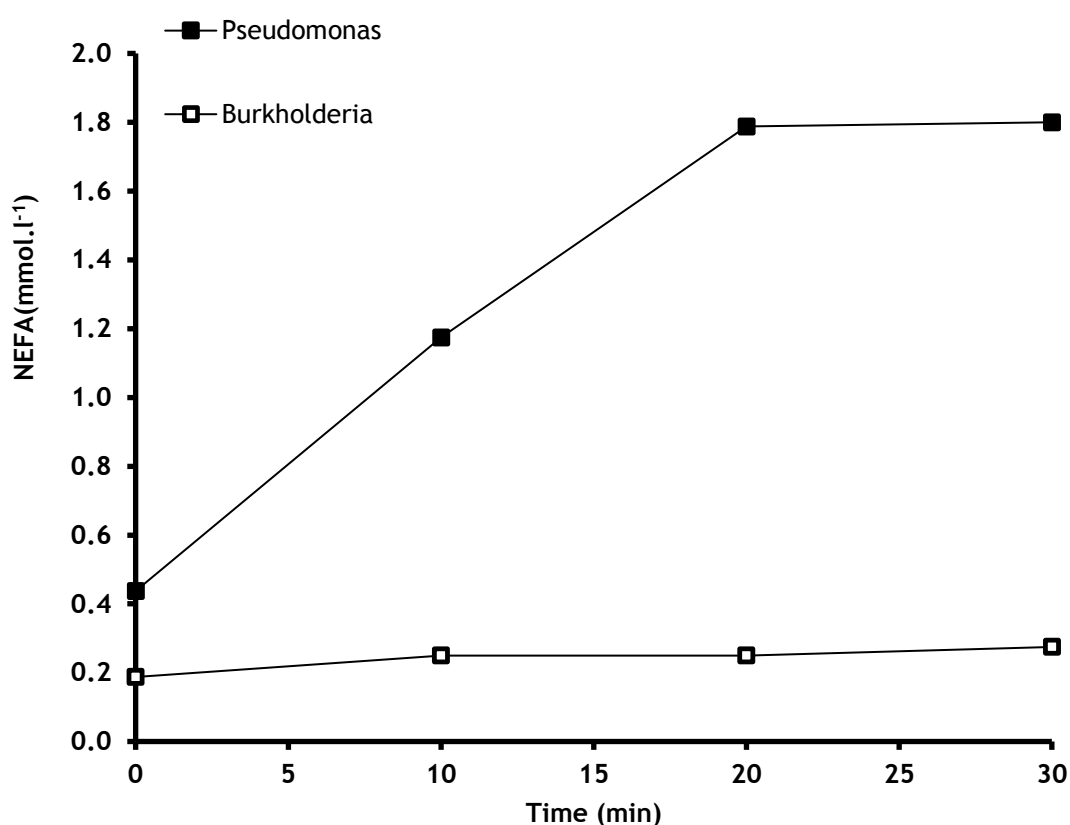


Figure 3.9. Release of NEFA over 30-minutes in LPL-affinity assay in VLDL₁ using 0.1 unit of LPL from different sources, *Burkholderia* sp. and *Pseudomonas* sp.

3.3.4 Conclusion

The combination of using 0.1 units of LPL from *Pseudomonas* sp. and reducing the final volume of the reaction mixture to 140 μ l led to an acceptable rate of NEFA release in the TG-hydrolysis assay. It was decided to use these conditions for the assay going forward.

3.4 Assay conditions

3.4.1 Stopping the reaction

There are many enzyme inhibitors that have been used to inhibit LPL action in different studies. Use of L- paraxon has been reported (Saheki et al., 1991), however its high toxicity meant that it was not considered for use here. Tetrahydrolipstatin (THL) has been used as an inhibitor of mammalian lipases, including pancreatic lipase, LPL, and HL (Hadvary et al., 1991, Lookene et al., 1994, Zambon et al., 1996). However, the THL solution is not completely clear, small particles may remain floating, which make it impractical to be used in the current assay. In addition, ethanol:chloroform:heptane (1.00.90.7, v/v) was used in a study by Shirai and Jackson to inhibit LPL activity (Shirai and Jackson, 1982, Gómez-Coronado et al., 1993). Again, chloroform is a poisonous liquid. Sodium chloride has also been used as an inhibitor of LPL previously (van Barlingen et al., 1996, Fielding and Fielding, 1976). In current study NaCl was used as the inhibitor, because it is practical and not poisonous. Based on the original paper by (van Barlingen et al., 1996) an initial concentration of NaCl of 2 M was chosen. To test whether this concentration was sufficient, a higher concentration of 5 M was also tested.

Results

Figure 3.10, Panel [A] shows NEFA concentrations when the reaction was quenched with 2 M and 5 M NaCl, with NEFA concentrations measured immediately after the assay was completed (i.e. at 30 minutes). **Figure 3.10, Panel [B]** shows the same data when NEFA concentrations were re-measured an hour later. There are two clear observations here. First, NEFA concentrations were higher at the

early time-points of the reaction when quenched with 2 M NaCl than with 5 M NaCl, suggesting that 2 M NaCl was not sufficient to quench the reaction and TG-hydrolysis was continuing until the NEFA measurement. Secondly, when the NEFA was re-measured one hour later, concentrations were higher compared with immediate NEFA measurement when the reaction was quenched with 2M NaCl, but not 5 M NaCl, which reinforces the initial observation that 2 M NaCl was not sufficient to fully quench the reaction, and demonstrates that 5 M NaCl was sufficient. Thus, a decision was made to use 5 M NaCl to quench the reaction.

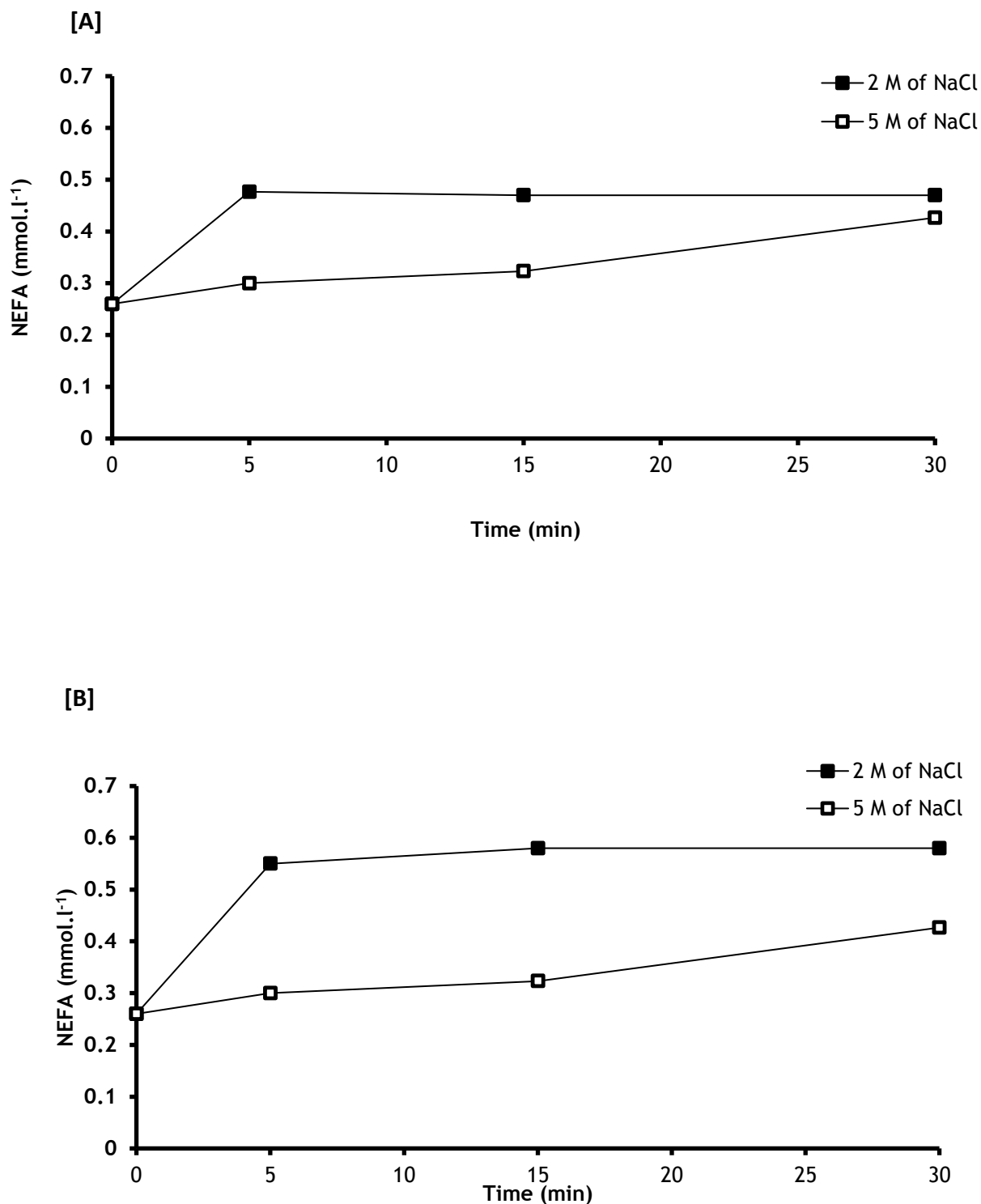


Figure 3.10. Stopping the reaction by using two concentrations of NaCl. Panel [A] shows the effect of adding 2 and 5 M of NaCl to stop the reaction, Panel [B] shows the repeated measurement for the same reaction after one hour.

3.5 Discussion

The purpose of the present study was to develop a method to measure lipolytic capacity of TRL, based on a previously published approach by (van Barlingen et al., 1996). Although, there are many methods to measure the lipolytic capacity for LPL, this assay is relatively easy to perform and meaningful in that it provides a good measure for the lipolytic rate in a given sample. There was some modification needed to reach to the optimal assay. Concentrating TRL helped to overcome any error from low concentrations measurements of NEFA. The modification of assay mixture and volume to avoid any dilutions related low concentrations. The LPL assay has been improved after the changes that had been made. The beginning was with ILab-600, but after exploring alternatives, and ILab-600 maintenance, automatic sample analysis using the ILab-600 presented the best option. Albumin removal enhanced the action of LPL to hydrolyse TG, with a more than 2 fold increase in detected NEFA, which is in line with a number of observations showing reduced NEFA recovery when albumin is present, using the same detection method (Duncombe, 1963, Duncombe, 1964, Matsubara et al., 1983). Moreover, the detection of TG concentration without albumin was better than with albumin, but these results were based on data from only one experiment. Repeats confirmed this observation. The final concentration of LPL was 0.1 units as it gives a linear increase of NEFA release during 30 minutes of interval. Before making all these modifications in the assay, a quality control was carried out thought out all the equipment that involved in the assay.

The final assay mixture ended up by using the following; 70 µl lipoprotein (0.6 mmol.l⁻¹) incubated with 35 µl of Tris-HCl buffer at 37 °C for 10 min to climate the tube. A 35 µl of LPL were added in each tube except the control, and incubated

for different time points 0, 5, 10, 15, 20 and 30 mins. Each tube was quenched by 35 µl of pre-cooled NaCl (5 M). NEFA was measured, in triplicate, at different time point using enzymatic colorimetric methods. The final reaction conditions are shown in **Table 3.3**. The protocol of the assay is illustrated in **Figure 3.11**. One limitation of this assay is that bacterial, rather than human, LPL was used, thus the absolute rates of TRL TG-hydrolysis are likely to differ from values obtained if human LPL was used. However, the purpose of the assay was to compare relative differences between susceptibility of TRL species to TG-hydrolysis under different experimental conditions and as the same type of LPL will be used under all conditions, the outcomes should be largely independent of the LPL species used. In conclusion, by concentrating TRL, removing albumin from the assay and lowering the assay volume, the measurements of both TG and NEFA become consistent and enabled this assay to be used in the study described in experimental **Chapter 4**.

Table 3.3; Final assay mixture.

	Time (min)							
	Control							
	0	0	0	5	10	15	20	30
All in μ l								
Buffer	35	70	35	35	35	35	35	35
Albumin	0	0	0	0	0	0	0	0
LPL	0	35	0	35	35	35	35	35
lipoprotein	0	0	70	70	70	70	70	70
Density sol.	70	0	0	0	0	0	0	0
Total Vol.	140	140	140	140	140	140	140	140
NaCl	93	93	93	93	93	93	93	93

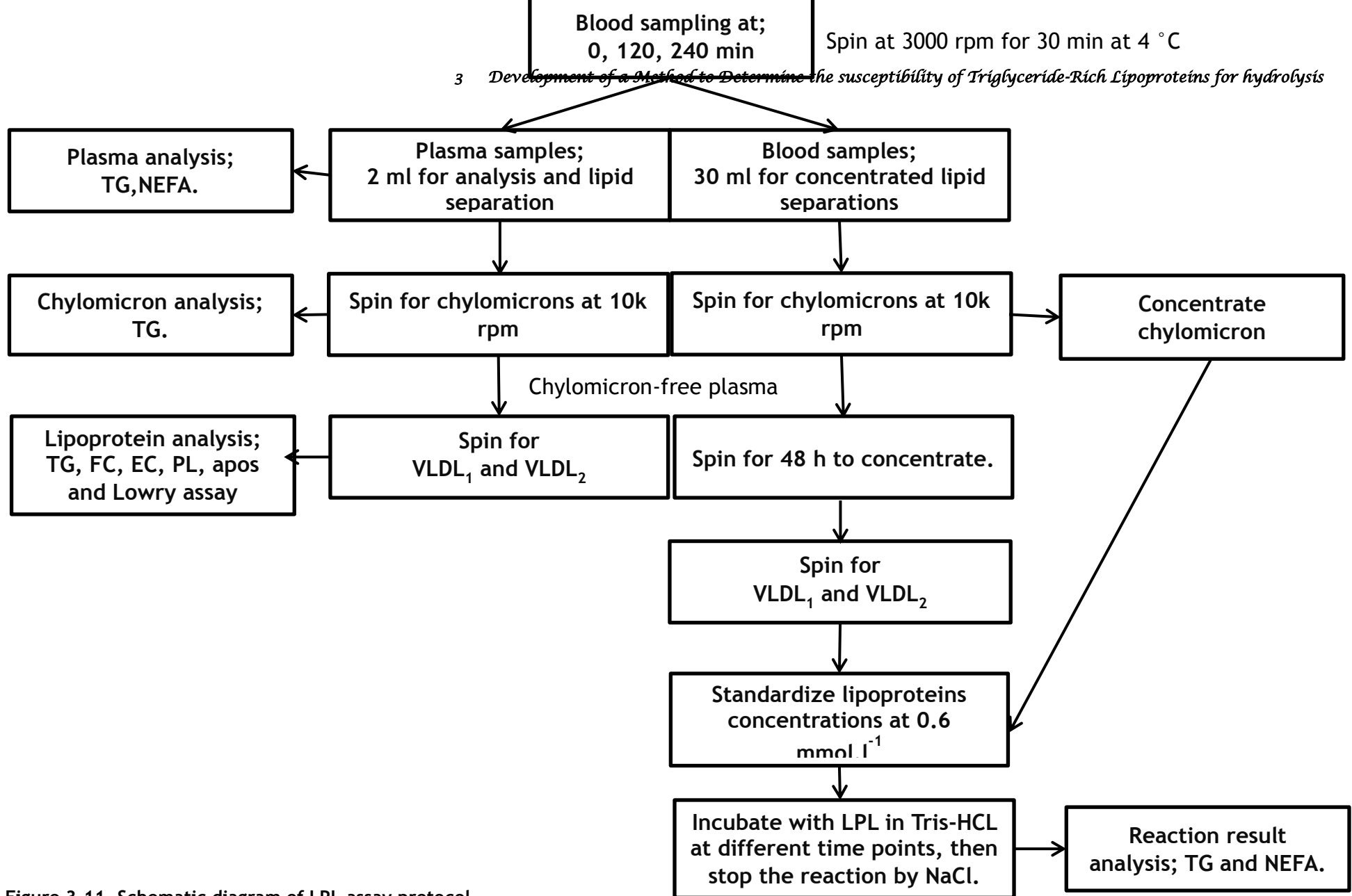


Figure 3.11. Schematic diagram of LPL assay protocol.

4 Effect of Exercise on the Affinity of Lipoproteins for Lipoprotein Lipase

4.1 Introduction

Postprandial triglyceride concentrations are independently associated with risk of cardiovascular events (Bansal et al., 2007, Mora et al., 2008, Nordestgaard et al., 2007) and chylomicrons and their remnants are implicated in the atherosclerotic disease process (Goldberg IJ, 2011, Chapman et al., 2011). Recent Mendelian randomisation studies reasserted the likely causal role of TG-mediated pathways in CVD (Jørgensen et al., 2012, Consortium and Collaboration, 2010, Cohorts, 2014, Jørgensen et al., 2014). Moderate intensity exercise lowers postprandial TG concentrations by about 15-25% in a range of population groups at increased risk of cardiovascular disease (Farah and Gill, 2012, Gill et al., 2004a, Gill and Hardman, 2000) and is recommended as a TG-lowering intervention for patients at high CVD risk (Chapman et al., 2011). However, the mechanism(s) by which exercise lowers TG have not been fully elucidated. The exercise-induced reduction in postprandial TG concentration is quantitatively greater in VLDL than in chylomicrons (Gill et al., 2006, Gill et al., 2001b), with large VLDL particles (VLDL₁, S_f 60- 400) being the lipoprotein subclass most affected (Gill et al., 2006). Kinetic studies have shown that exercise-induced VLDL-TG reductions are due to increased clearance from the circulation, rather than reduced hepatic production (Al-Shayji et al., 2012). While exercise has also been shown to increase clearance of chylomicron-like particles (Al-Shayji et al., 2012, Sady et al., 1986, Annuzzi et al., 1987), the magnitude of this change is smaller than the increase in clearance of VLDL₁-TG (Al-Shayji et al., 2012), and the effect has not been consistently observed (Gill et al., 2001a). Furthermore, although exercise-induced reductions in postprandial TG concentrations are sometimes accompanied by an increase in post-heparin plasma or skeletal muscle lipoprotein lipase (LPL) activity, post-exercise TG reductions are also commonly observed in the absence of increased LPL activity (Harrison et al., 2012, Herd et al., 2001). The affinity of chylomicrons/chylomicron-like particles for LPL clearance is many fold greater than that of VLDL particles (Björkegren et al., 1996); thus the observation

that exercise increases VLDL₁-TG clearance to a greater extent than chylomicron-like particles (Al-Shayji et al., 2012), taken together with the inconsistent changes to LPL activity in response to exercise (Malkova et al., 2000), suggest that mechanisms other than increased LPL activity are likely to contribute to the exercise-induced increase in VLDL₁-TG removal. There is evidence that circulating VLDL₁ particles are larger and more TG enriched following exercise (Al-Shayji et al., 2012, Gill et al., 2006) changes that might be expected to increase the susceptibility (affinity) of these particles for LPL-mediated hydrolysis (Fisher et al., 1995) and we have recently demonstrated that these exercise-induced changes to the size and TG enrichment of circulating VLDL₁ particles explain about half of the variance in the exercise-induced increase in VLDL₁ particle clearance in correlational analyses (Al-Shayji et al., 2012). This observation is consistent with exercise-induced changes to VLDL₁ particles increasing their affinity for LPL-mediated clearance (Magkos, 2009, Al-Shayji et al., 2012): this data interpretation would also explain how exercise could increase clearance of VLDL₁-TG without necessarily increasing LPL activity, and why the exercise-induced increase in VLDL₁-TG clearance is quantitatively larger than that observed in chylomicron-like particles. However, the hypothesis that exercise increases the affinity of VLDL₁ as a substrate for LPL has not been directly tested. The purpose of this study was therefore to determine the effects of exercise on the affinity of TG-rich lipoprotein species (chylomicrons, VLDL₁, and VLDL₂ (S_f 20-60)) for LPL-mediated TG hydrolysis.

4.2 Participants and Methods

4.2.1 Participants

Fifteen overweight/obese men were initially recruited to this study. Three were excluded at the outset for not being normoglycaemic. A further two withdrew without completing the experimental period for undisclosed personal reasons. Characterizations of the remaining 10 are listed in **Table 4.1**. All subjects were apparently healthy, normotensive, normoglycaemic and nonsmokers. None was taking any drugs known to affect lipid or carbohydrate metabolism. The study was

conducted with the approval of University of Glasgow Ethics Committee, and subjects gave written informed consent prior to participation

Table 4.1; Physical characteristics

Age (years)	36.44 ± 0.77
Weight (kg)	95.85 ± 24.39
Height (m)	1.76 ± 0.09
BMI (kg.m ⁻²)	30.46 ± 6.27
Waist (cm)	101.85 ± 15.40
Percentage fat (%)	26.66 ± 5.84
Sum of skinfolds* (mm)	56.27 ± 42.61
Systolic blood pressure (mm Hg)	127.09 ± 17.03
Diastolic blood pressure (mm Hg)	81.27 ± 10.62

N=10 Values are means ± SD

*triceps, biceps, subscapular and superiliac

4.2.2 Study design

Participants attended the laboratory for two oral fat loading test (FLT) in a random order, with an interval of 7-14 days. On the day before one of the OFTTs, participants walked on a treadmill for 90-min at an intensity of 50% VO₂max (Exercise trial). For the other FLT, subjects performed no exercise on the day preceding the oral fat tolerance test (Control trial). Subjects were asked to weigh and record their dietary intake and refrain from alcohol for the 2 days prior to the first FLT and replicated this prior to the second fat tolerance test. During the 3 days prior each FLT subjects were instructed to perform no exercise, other than the treadmill walk in the exercise trial (see Figure 4.1).

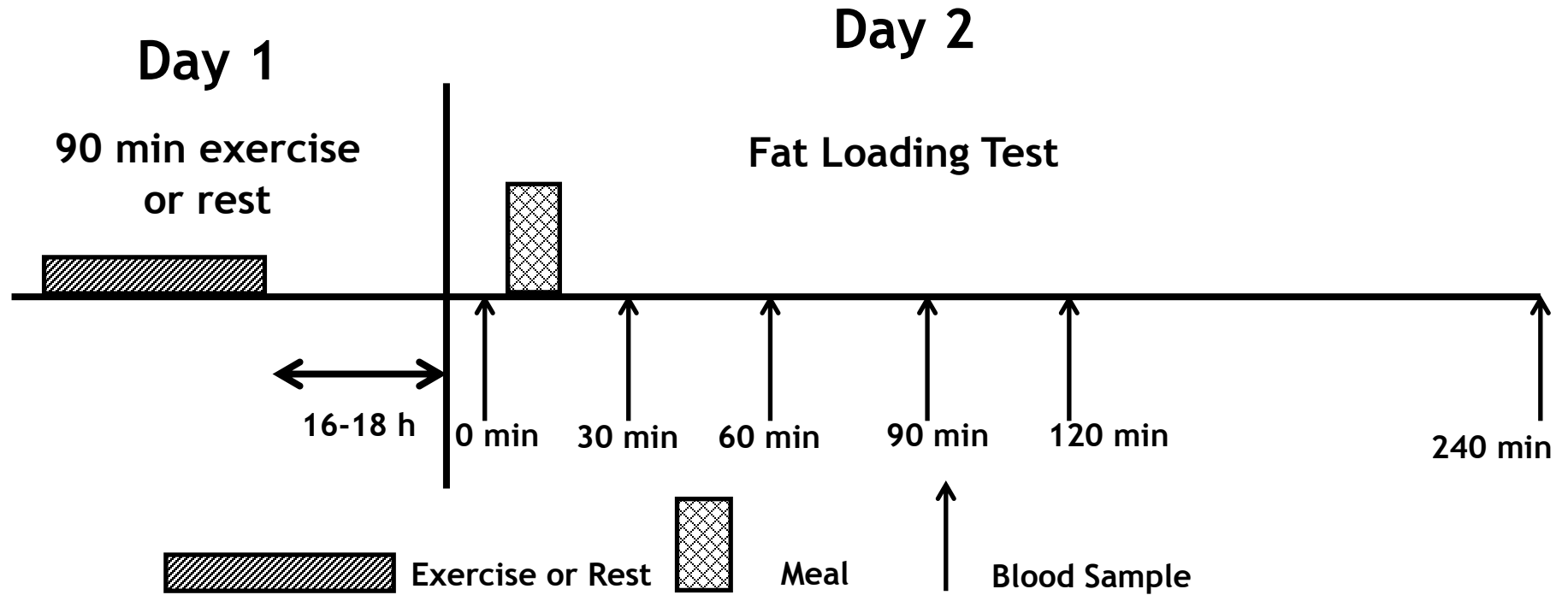


Figure 4.1. Study Design. A day prior the OFTT participants performed either exercise for 90 minutes or rest. A base line blood sample was acquired, then a test meal was provided, further blood samples were obtained at 30, 60, 90, 120 and 240 minutes after meal consumption.

Exercise tests

One week prior the first FLT a preliminary sub-maximal incremental treadmill test was performed to estimate $\dot{V}O_{2\max}$ (Armstrong, 2006) and determine the walking speed and gradient required to elicit 50% $\dot{V}O_{2\max}$ as mentioned in **section 2.4**. In the exercise trial, the treadmill walk was performed on the afternoon preceding the oral fat tolerance test about 16-18 h before the FLT. During the walk, O_2 uptake and CO_2 production were measured as mentioned in **section 2.2**, heart rate was measured by short range telemetry (Polar Electroky, Kempele, Finland) as mention in **section 2.3** and ratings of perceived exertion (Borg, 1973) were obtained at 15-minute intervals.

Fat loading test

Subjects reported to the lab in the morning after an overnight fast for ≥ 12 h. A cannula was placed in an antecubital vein and, after a 10-min interval, a fasted state blood sample was withdrawn. A high fat mixed meal (HFM) as mentioned above in **section 2.5.1** was provided. Further blood samples were obtained at 30, 60, 90, 120 and 240 minutes after meal consumption. Subjects rested and consumed only water during this time.

4.2.3 Lipoprotein separation

Plasma samples (2 ml) at 0, 2 and 4 h were centrifuged to isolate lipoprotein subfractions as previously described in **section 2.6.2** VLDL₁ and VLDL₂ fractions were assayed to determine concentrations and composition for TG, free cholesterol (FC) and phospholipids (PL), apoB, apoE and apoC as mention in **Appendix C**. Cholesteryl ester (CE) was determined by using the equation mentioned in **Appendix C**. Chylomicron fractions were assayed for TG concentration. In the VLDL₁ and VLDL₂ fractions, total protein was measured using a modified Lowry assay (see **section 2.7**).

At the 4 h time point, chylomicrons were separated from 30 ml plasma by centrifugation. The chylomicron fraction was then concentrated 8-10 fold by centrifugation as mentioned in **section 3.2**. The chylomicron-free plasma at the 4 h time-point, and 10 ml of plasma collected at 0 hours was then concentrated

~10-fold by centrifugation (see **section 3.2**) before lipoprotein separation to obtain concentrated VLDL₁ and VLDL₂ fractions. Chylomicron concentrations were adjusted to 0.6 mmol.l⁻¹ of triglyceride, and VLDL₁ and VLDL₂ concentrations were adjusted to 0.6 and 2.0 mmol.l⁻¹, by the addition of a d 1.006 g.ml⁻¹ density solution.

4.2.4 LPL affinity assay

Affinity of lipoproteins for LPL was determined using a modified version a method described previously as mention in **Chapter 3**.

4.2.5 Plasma assays

Plasma glucose, TG, NEFA, and 3-hydroxybutyrate concentrations were analysed at all time points as previously mentioned in **Appendix C**. Total, HDL cholesterol concentrations were measured in the fasted state as mentioned in **Appendix C**. Small dense LDL was measured in the fasted state and 4 h postprandially as mention in **Appendix C**, LDL cholesterol was calculated in the fasted state using the Friedewald equation (Friedewald et al., 1972). Insulin was measured in EDTA plasma as described in **section 2.6.4**.

4.2.6 Data analysis

Statistical analyses were performed using Statistica (version 10, StatSoft Inc.) and Minitab (version 17, Minitab Ltd). All data were tested for normality using the Anderson-Darling test. Where data did not approximate a normal distribution, these were log-transformed prior to analysis and data are expressed as geometric means with 95% confidence intervals (95% CI)(Bland and Altman, 1996a, Bland and Altman, 1996b). Time-averaged postprandial concentrations, calculated as the trapezium rule-derived areas under concentration versus time curve, divided by the duration of the postprandial observation period (240 minutes), were used as summary measures of the postprandial responses. Comparisons between trials were made using paired *t*-tests where a single pair of means was compared, or by two-way repeated measures ANOVA, with *post hoc* Fisher tests, when comparisons were made across multiple time-points. Differences in lipoprotein affinity for LPL were expressed in terms of absolute changes and in terms of relative (fold)

changes. Relationships between variables were assessed using Pearson product-moment correlations. Cohen's effect size was calculated to measure the magnitude of the exercise effect (Cohen, 2013) see section 2.9.5. Statistical significance was accepted at $p < 0.05$. Please see Appendix E for Dr.Farag Al-Shuweihdi statistical report.

4.3 Results

4.3.1 Plasma concentrations in the fasted and postprandial states

Plasma TG concentrations in the fasted and postprandial states for the group are shown in **Figure 4.2** and **Tables 4.2** and **4.3**. Individual values are shown in **Figure 4.3**. Exercise reduced fasting TG concentrations in 8, and postprandial TG in 7, of the 10 participants (**Figure 4.3, panels A and B**). TG concentrations were not normally distributed in either the fasted or postprandial states, largely due to one participant having substantially raised TG concentrations (> twice the mean) (see **Figure 4.3, panels A and B**) and thus statistical analysis was performed on log transformed data. Log transformed values approximated a normal distribution (**Figure 4.3, panels C and D**). Fasting TG concentrations were reduced by 18% (-0.18 (-0.20 to -0.04) mmol.l^{-1} (mean difference (95% confidence interval)); $p=0.04$) by exercise, to give a Cohen's d effect size for the exercise effect of 0.77. Time-averaged postprandial TG concentrations were reduced by 13% (-0.33 (-0.63 to -0.04) mmol.l^{-1} ; $p=0.04$), to give a Cohen's d effect size of 0.70. Exercise had a statistically significant, moderate-sized effect on TG concentrations in both the fasted and postprandial states (Cohen, 2013).

Group data for insulin are shown in **Figure 4.4, panel B** and **Tables 4.2** and **4.3**. Like TG, fasting and postprandial insulin concentrations were not normally distributed due to one individual with substantially elevated concentrations (see **Figure 4.5, panels B and D**), so values were log transformed prior to analysis. Although exercise reduced mean fasting insulin concentrations by ~25% and postprandial insulin concentrations by almost 60%, there was substantial individual variability in the exercise response (6 out of 10 lower with exercise in both fasted and postprandial states), leading to non-significant effects of exercise on both fasting (mean (95%CI) difference: -0.29 (-0.34 to $+0.26$) mU.l^{-1}) and postprandial (-0.15 (-0.19 to $+0.24$) mU.l^{-1}) insulin concentrations. Also, 3-hydroxybutyrate values were not normally distributed due to one individual with substantially elevated concentrations (see **Figure 4.7, panel C** and panel D) therefore, values were log transformed prior to analysis. Fasted 3-hydroxybutyrate values were higher in the exercise trial by 77% $+0.37$ ($+0.34$ to $+0.62$) mmol.l^{-1} ; $p=0.02$) but

there was no significant difference between the two trials in the postprandial state (-0.08 (-0.12 to +0.36) mmol.l⁻¹; p=0.74). The Cohen's d effect size was 0.89 and 0.11 respectively.

Figures 4.7, panels A and C, Figure 4.8, panels A and C and Tables 4.2 and 4.3 show fasting and postprandial concentrations for glucose and NEFA control and exercise trials. NEFA did not significantly change by exercise, although there was a trend for fasting values for NEFA (+0.07 (-0.01 to +0.15) mmol.l⁻¹) to be higher in the exercise trial.

Exercise did not significantly affect total, LDL or HDL cholesterol concentrations in the fasted state (**Table 4.2**), but fasted small dense LDL concentrations were significantly lower by 16% (-0.23(-0.31 to -0.16) mmol.l⁻¹ (mean difference (95% confidence interval); p=0.0002) following exercise, to give a Cohen's d effect size for the exercise effect of 1.89. Time-averaged postprandial sdLDL concentrations were reduced by 11% (+0.17 (-0.13 to +0.47)) mmol.l⁻¹; p=0.0006), to give a Cohen's d effect size of 0.35. (see **Figure 4.8, panel A and panel B and Table 4.2 and 4.3**).

Table 4.2; Fasting plasma concentrations.

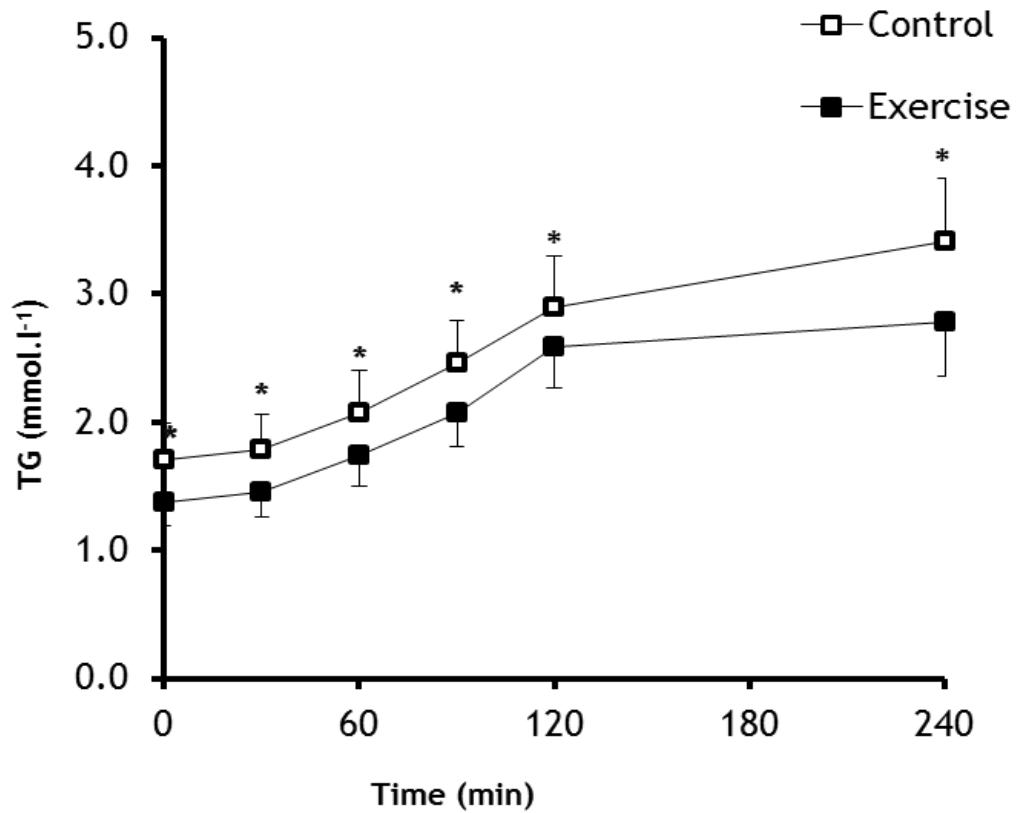
	Fasting concentration				p-value
	Control	Exercise	Mean difference and 95% CI	Cohn's <i>d</i> effect size	
Plasma TG (mmol.l ⁻¹)*	0.45 ± 0.14	0.27 ± 0.15	-0.18 (-0.20 to -0.04)	0.77	0.04
Glucose (mmol.l ⁻¹)	5.50 ± 0.22	5.47 ± 0.23	-0.04 (-0.31 to +0.24)	0.08	0.81
Insulin (mU.l ⁻¹)*	2.50 ± 0.35	2.21 ± 0.20	-0.29 (-0.34 to +0.26)	0.33	0.33
NEFA (mmol.l ⁻¹)	0.66 ± 0.06	0.73 ± 0.05	+0.07 (-0.01 to +0.15)	0.57	0.11
3-hydroxybutyrate (mmol.l ⁻¹)*	2.45 ± 0.25	2.82 ± 0.21	+0.37 (+0.34 to +0.62)	0.89	0.02
Plasma Small dense LDL (mmol.l ⁻¹)	1.40 ± 0.20	1.17 ± 0.17	-0.23 (-0.31 to -0.16)	1.89	0.0002
Total cholesterol (mmol.l ⁻¹)	5.44 ± 0.33	5.49 ± 0.36	+0.05 (-0.19 to +0.28)	0.13	0.69
HDL cholesterol (mmol.l ⁻¹)	1.06 ± 0.09	1.09 ± 0.10	-0.03 (-0.09 to +0.03)	0.29	0.38
LDL cholesterol (mmol.l ⁻¹)	3.68 ± 0.28	3.83 ± 0.33	+0.15 (-0.06 to +0.35)	0.45	0.19

Values are mean ± SEM, n = 10. Statistical analysis performed using paired t-test. *log-transformed values.

Table 4.3; Time-averaged postprandial plasma concentrations.

	Postprandial concentration				
	Control	Exercise	Mean difference and 95% CI	Cohen's <i>d</i> effect size	p-value
Plasma TG (mmol.l ⁻¹)	2.72 ± 0.40	2.36 ± 0.31	-0.33 (-0.63 to -0.04)	0.70	0.03
Glucose (mmol.l ⁻¹)	6.39 ± 0.46	6.35 ± 0.36	-0.04 (-0.34 to +0.33)	0.08	0.81
Insulin (mU.l ⁻¹)*	4.24 ± 0.27	4.09 ± 0.25	-0.15 (-0.19 to +0.24)	0.24	0.33
NEFA (mmol.l ⁻¹)	0.65 ± 0.06	0.64 ± 0.06	-0.01 (-0.09 to +0.07)	0.005	0.11
3- hydroxybutyrate (mmol.l ⁻¹)	0.08 ± 0.01	0.10 ± 0.03	-0.02 (-0.09 to +0.04)	0.24	0.74
Plasma Small dense LDL (mmol.l ⁻¹)	1.31 ± 0.16	1.14 ± 0.14	+0.17 (-0.13 to +0.47)	0.35	0.0002

Values are mean ± SEM, n = 10. Statistical analysis performed using paired t-test. *log-transformed values. Statistical analysis performed using paired t-test



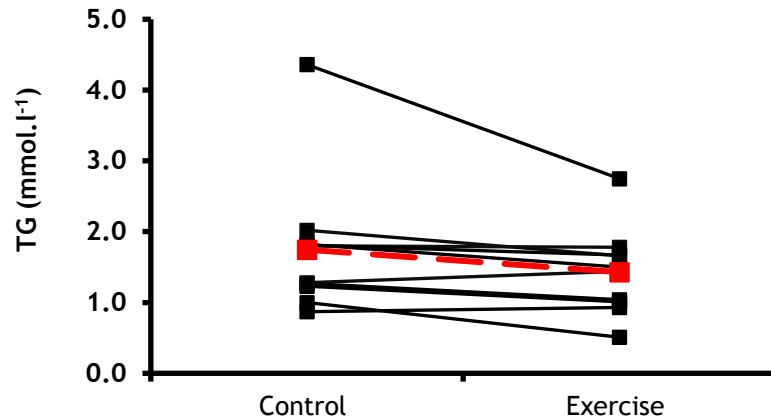
Time	0 min	30 min	60 min	90 min	120 min	240 min
Mean difference	0.32* ± 0.16	0.29* ± 0.15	0.31* ± 0.13	0.37* ± 0.14	0.26* ± 0.19	0.57* ± 0.19

Figure 4.2. Time-averaged postprandial plasma TG concentrations in Control and Exercise trial. Values and statistical analysis of these data is shown in Table 4.1 and 4.2. N = 10, Values are mean ± SEM. The SEM shows the variability among the whole population.

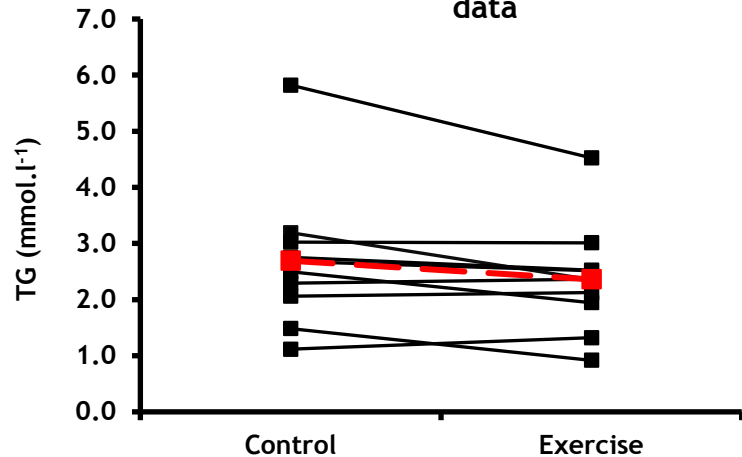
The table below, shows the mean ± SEM differences between the two trials at each time point.* significant difference between trials at this time-point within subject ($p < 0.005$).

Note. Error bars are calculated on the between subjects data, but the test is of the within subjects data.

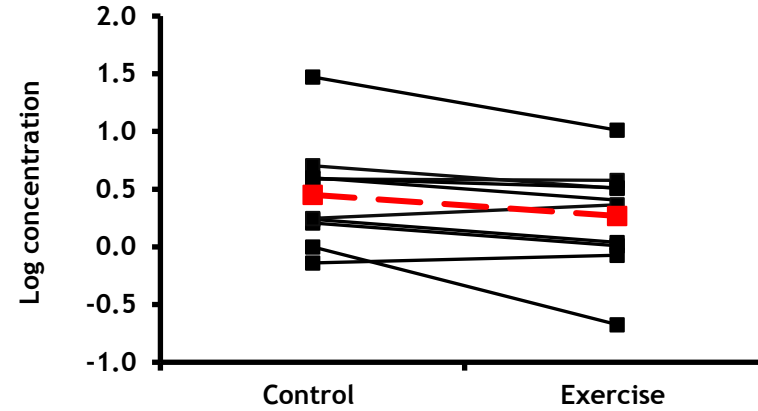
[A] Fasted concentrations - raw data



[B] Time-averaged postprandial concentrations - raw data



[C] Fasted concentrations - log transformed data



[D] Time-averaged postprandial concentrations - log transformed data

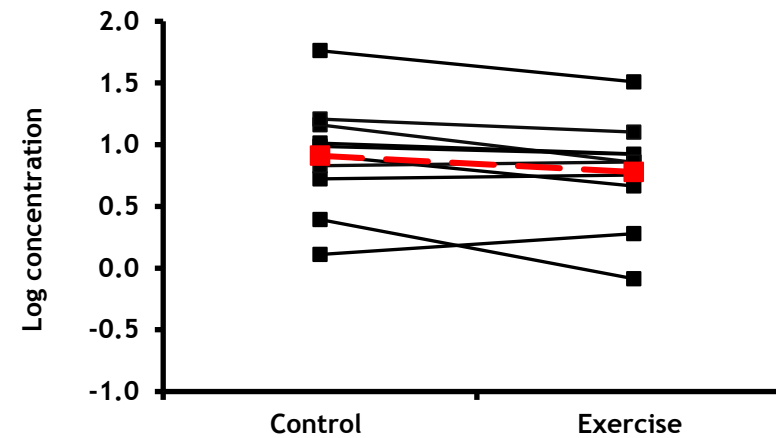


Figure 4.3. Individual values for TG in the Control and Exercise trials. Panel [A] shows raw values for fasting TG concentrations; panel [B] shows raw values for time-averaged postprandial TG concentrations; panel [C] shows log transformed values for fasting TG concentration; panel [D] shows log-transformed values for time-averaged postprandial TG concentrations. Black symbols show individual values; red symbols show the mean values. Values and statistical analysis of these data is shown in Table 4.2 and Table 4.3.

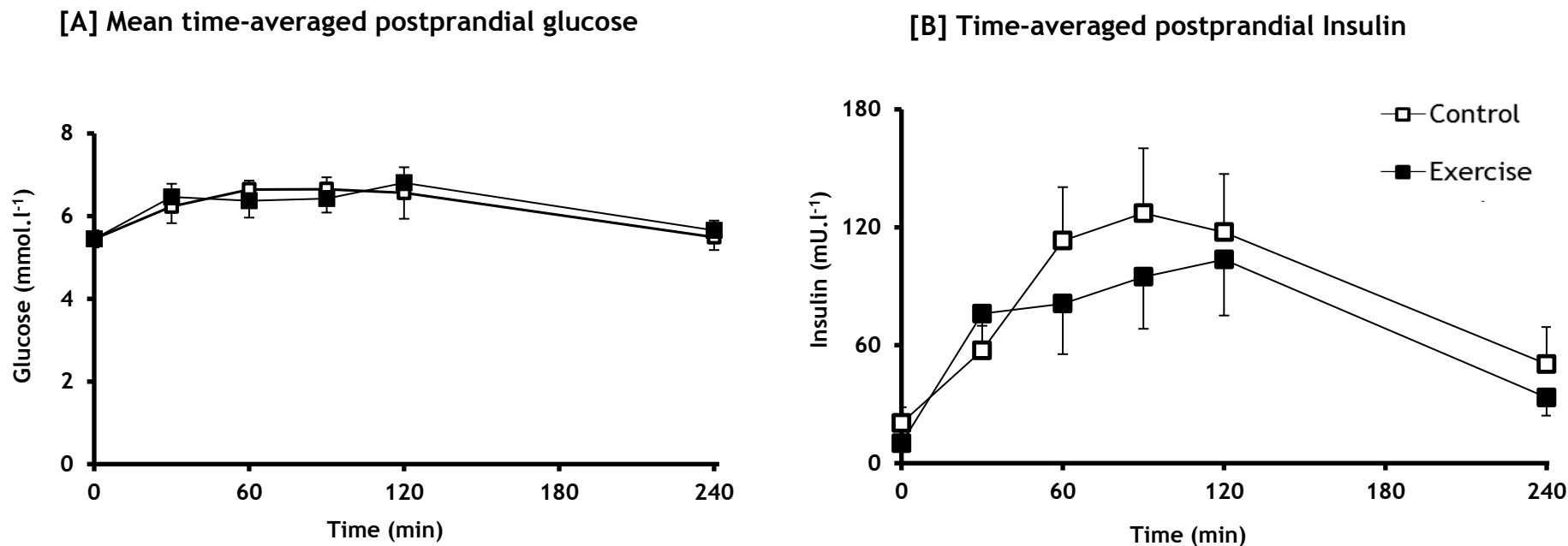


Figure 4.4. Time-averaged postprandial concentrations in Control and Exercise trial, panel [A] shows time-averaged postprandial glucose concentrations and panel [B] shows time-averaged postprandial insulin concentrations. Values and statistical analysis of these data is shown in Table 4.2 and Table 4.3 and 4.2 N = 10, Values are mean \pm SEM. The SEM shows the variability among the whole population. * significant difference between trials at this time-point within subject ($p < 0.005$).

Note. Error bars are calculated on the between subjects data, but the test is of the within subjects data.

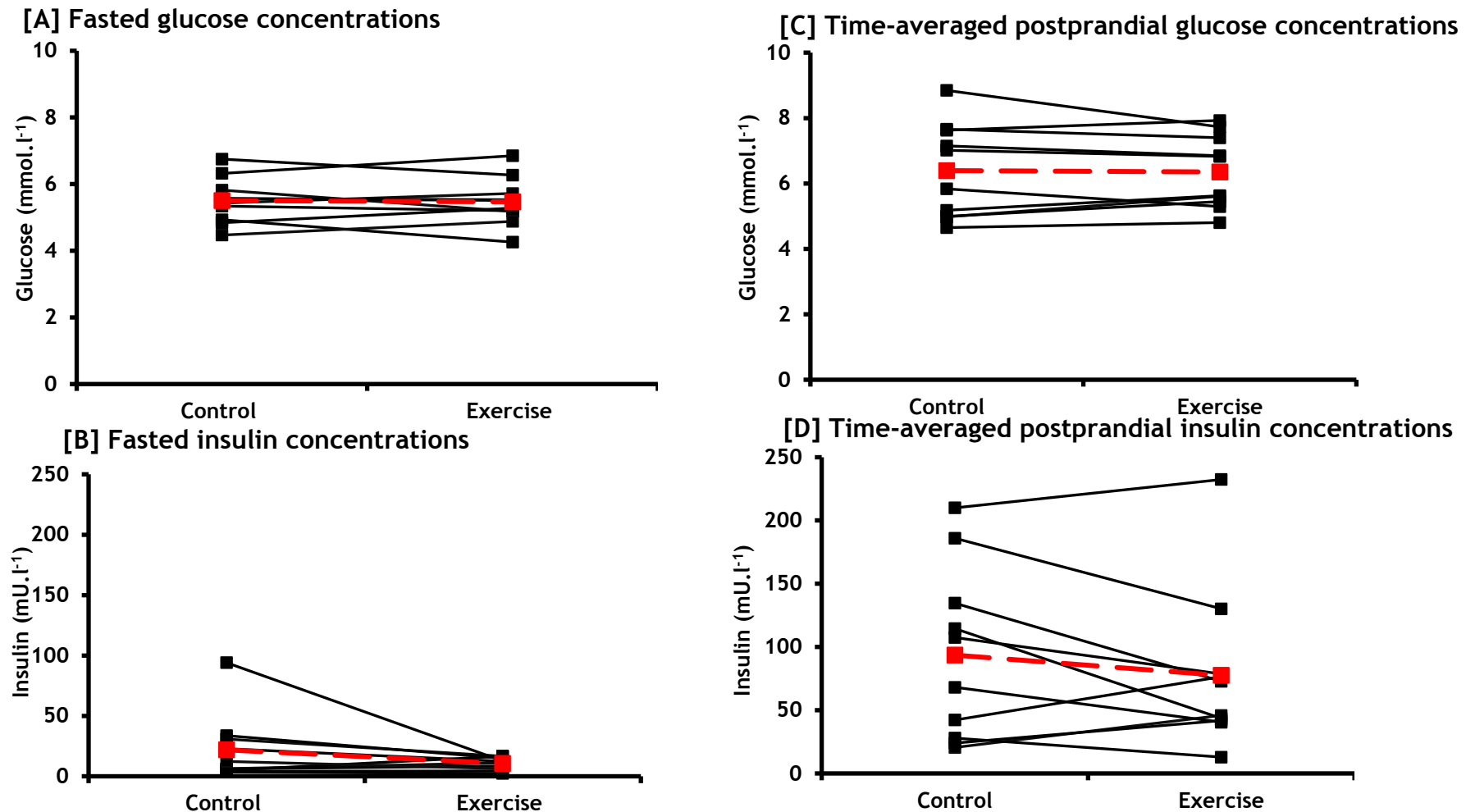


Figure 4.5. Individual values for glucose and insulin in the Control and Exercise trials. Panel [A] shows raw values for fasting glucose concentrations; panel [B] shows fasting insulin concentrations; panel [C] shows time-averaged glucose concentration; panel [D] shows time-averaged postprandial insulin concentrations. Black symbols show individual values, red symbols show the mean values. Values and statistical analysis of these data is shown in Table 4.2 and Table 4.3.

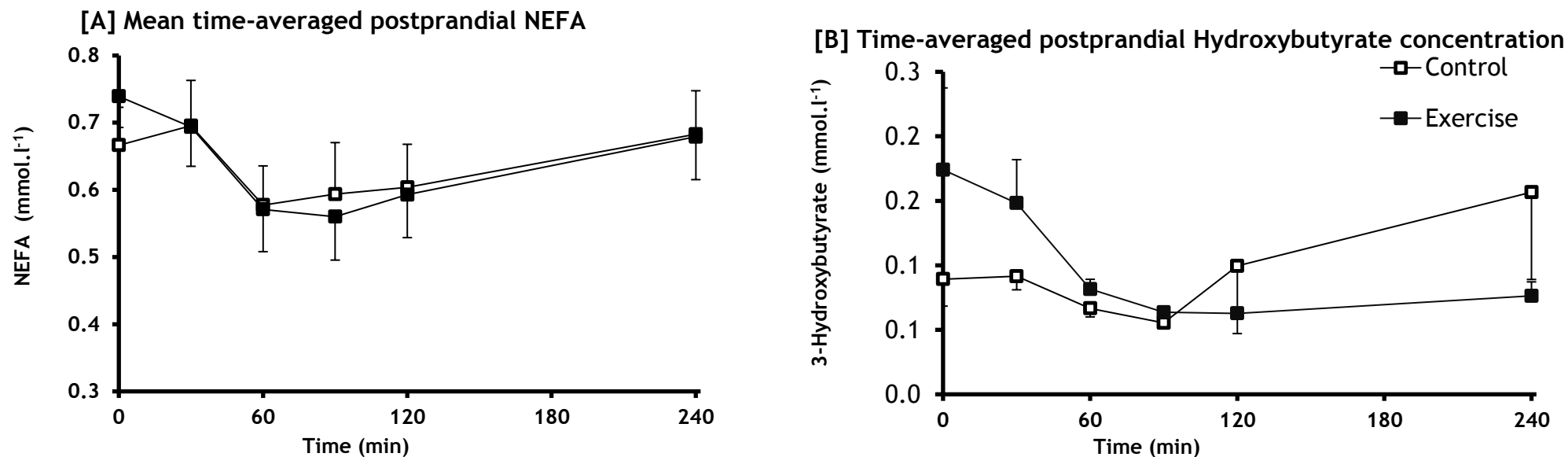


Figure 4.6. Time-averaged postprandial concentrations in Control and Exercise trial, panel [A] shows time-averaged postprandial NEFA concentrations and panel [B] shows time-averaged postprandial 3-Hydroxybutyrate concentrations. Values and statistical analysis of these data is shown in Table 4.2 and Table 4.3 and 4.2 N = 10, Values are mean \pm SEM. The SEM shows the variability among the whole population. * significant difference between trials at this time-point within subject ($p < 0.005$).

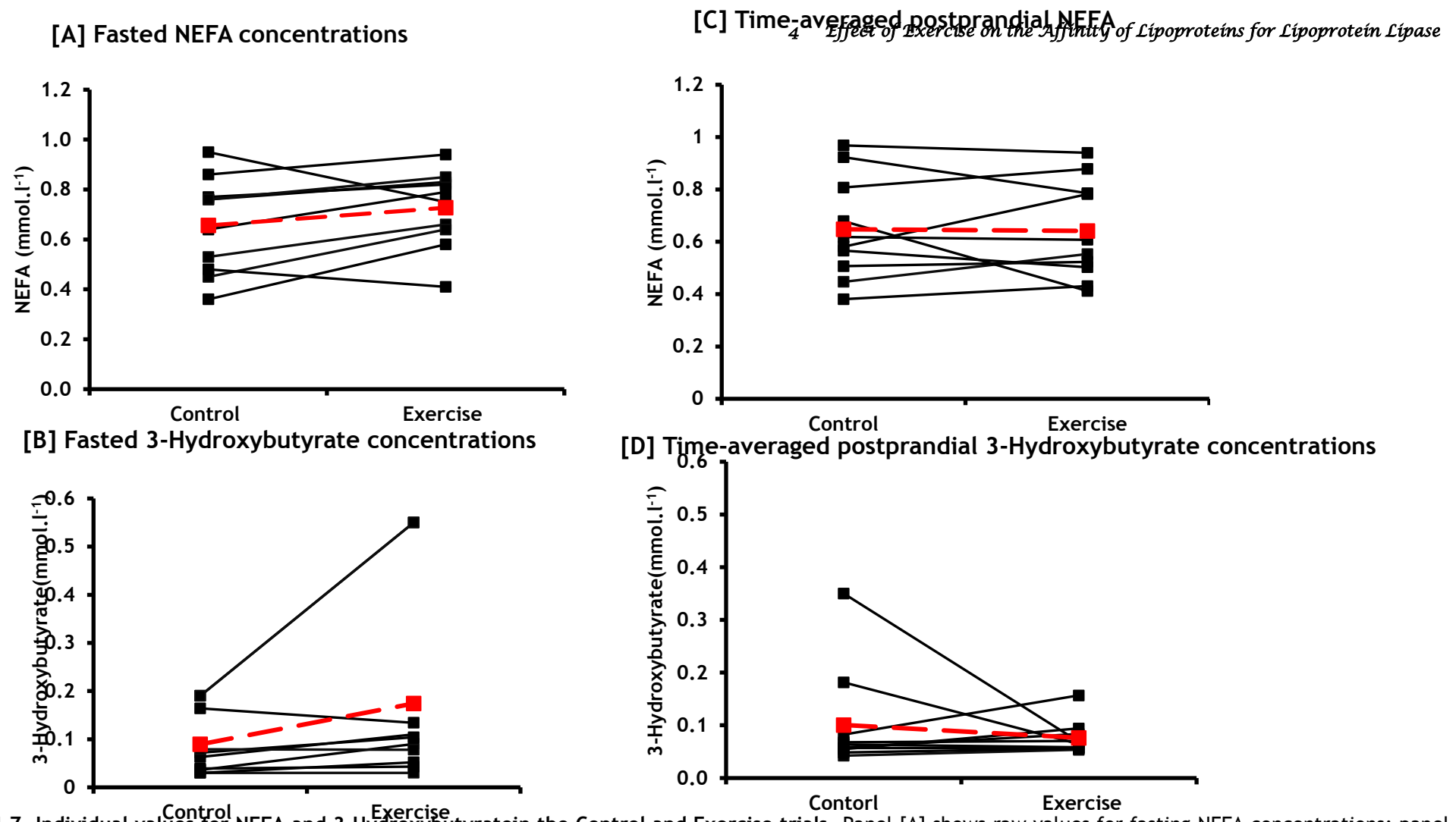


Figure 4.7. Individual values for NEFA and 3-Hydroxybutyrate in the Control and Exercise trials. Panel [A] shows raw values for fasting NEFA concentrations; panel [B] shows fasting 3-Hydroxybutyrate concentrations; panel [C] shows time-averaged NEFA concentration; panel [D] shows time-averaged postprandial 3-Hydroxybutyrate concentrations. Black symbols show individual values, red symbols show the mean values. Values and statistical analysis of these data is shown in Table 4.2 and Table 4.3.

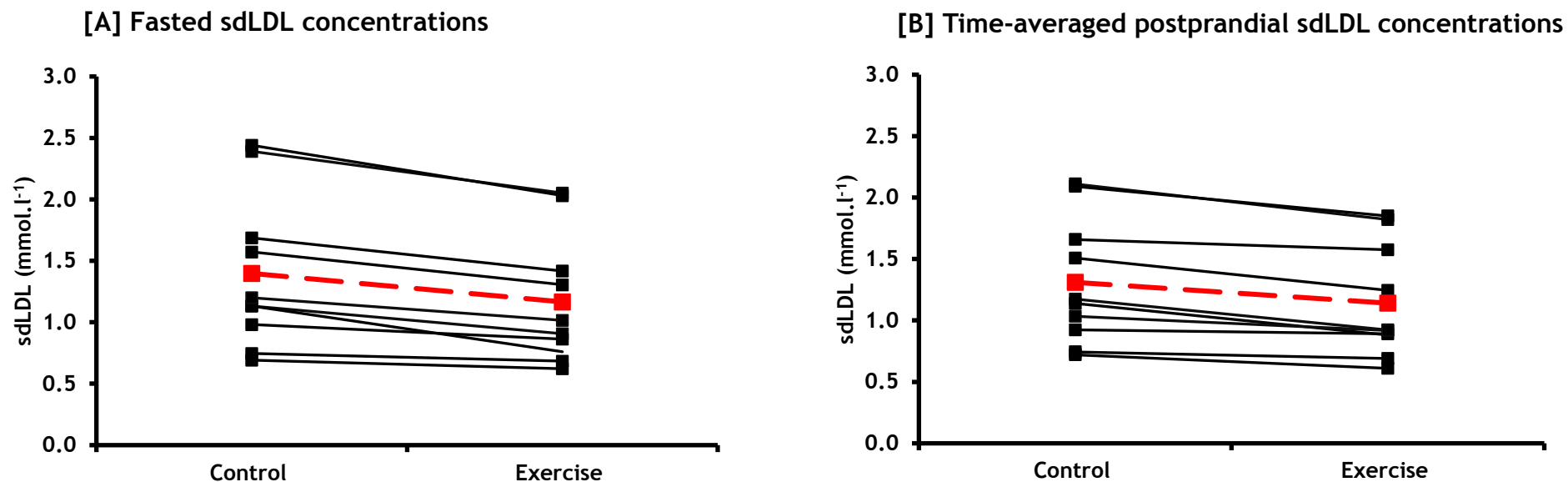


Figure 4.8. Individual values for sdLDL the Control and Exercise trials. Panel [A] shows fasting sdLDL concentrations; panel [B] shows time-averaged sdLDL concentration. Black symbols show individual values, red symbols show the mean values. Values and statistical analysis of these data is shown in Table 4.2 and Table 4.3.

4.3.2 Lipoprotein concentrations and composition in the fasted and postprandial states

Fasting and postprandial chylomicron-TG, VLDL₁-TG and VLDL₂-TG concentrations in the fasted and postprandial states for the group are shown in **Figure 4.9, panel A**, **Figure 4.10, panel A** and **Figure 4.11, panel A** and **Tables 4.4** and **4.5**. Individual values are shown in **Figure 4.9, panel B**, **Figure 4.10, panel B** and **panel C** and **Figure 4.11, panel B** and **panel C**. Chylomicron-TG concentrations in the fasted state were negligible in both trials, (-0.01 (-0.02 to +0.01) mmol.l⁻¹ (mean difference (95% confidence interval)); p=0.25) by exercise. Whereas, exercise reduced postprandial chylomicron-TG concentrations by 31% (-0.12 (-0.13 to +0.02); p=0.08). The reduction of chylomicron TG observed in 7 out of the 10 participants. The effect of exercise on chylomicron time averaged TG concentrations in postprandial state gave a Cohen's d effect size of 0.73 (see **Figure 4.9, panel B**). Exercise reduced fasting and postprandial VLDL₁-TG concentrations in 7 out of the 10 participants in both trials (**Figure 4.10, panel B** and **C**). Fasting VLDL₁-TG concentrations were reduced by 25% (-10.63 (- 21.26 to +0.00) mg.dl⁻¹ (mean difference (95% confidence interval); p=0.056)) by exercise, to give a Cohen's d for the exercise effect of 0.69. Time-averaged postprandial VLDL₁ TG concentrations were reduced by 18% (-14.62 (-27.40 to -1.84) mg.dl⁻¹; p=0.01), to give a Cohen's d effect size of 0.96. Thus, exercise had a statistically significant, moderate to large-sized effect on TG concentrations in both the fasted and postprandial. Exercise did not significantly affect fasted and time-averaged postprandial VLDL₂-TG concentration did not differ significantly between trials (-3.21(-8 .84 to +2.42); p=0.22) and (+0.44 (-3.98 to +4.86); p=0.10) mg.dl⁻¹ respectively. (see **Figure 4.11, panel B** and **C** and **Table 4.6** and **4.7**).

Table 4.4, Table 4.5, Table 4.6 and Table 4.7 show total lipoprotein particle concentration and concentration of constituent lipoprotein molecules for VLDL₁ and VLDL₂ in the fasted state and 240 minutes postprandially. Exercise reduced fasting and postprandial total VLDL₁ mass concentrations in 8 out of the 10 participants in both fasted and postprandial state. Fasting total lipoprotein mass for VLDL₁ was lower in the fasted state by 24% (-20.43 mg/dl (-39.34 to -1.51); $p = 0.04$) by exercise, to give a Cohen's d for the exercise effect of 0.79 and lower at 240 minutes postprandially by 17% (-23.34mg/dl (-45.83 to -0.86); $p = 0.049$), by exercise, to give a Cohen's d for the exercise effect of 0.69.

VLDL₁ apoB concentrations were significantly lower in the exercise than the control trial in the fasted state, but not 240 minutes postprandially. Concentrations of VLDL₁ TG, cholesteryl ester and free cholesterol were lower in the exercise than the control trial in both the fasted state and 240 minutes postprandially (Table 4.4 and 4.5). In the fasted state the VLDL₁ TG/apoB ratio tended to be 39% higher (6942 (-1097 to +14982); $p=0.09$) by exercise, to give a Cohen's d for the exercise effect of 0.60. At the 240 minute postprandial time-point VLDL₁ TG/apoB ratio was similar in the two trials. Similarly, in the fasted state, the CE/TG ratio tended to be 26% lower -0.06 (-0.15 to +0.03) ($p=0.16$). At the 240 minute postprandial time-point VLDL₁ CE/TG ratio was similar in the two trials. In contrast to the findings for VLDL₁, no differences in VLDL₂ concentration or composition were observed between the control and exercise.

Table 4.4; Concentration and composition of VLDL₁ in the fasted states.

	Fasting (0 minutes)				
	Control	Exercise	Mean difference and 95 % CI	Cohen's <i>d</i> effect size	p-value
Total lipoprotein concentration (mg.dl ⁻¹)	84.1 ± 17.4	63.7 ± 13.0	-20.43 (-39.34 to -1.51)	0.75	0.04
ApoB (mg.dl ⁻¹)	1.98 ± 0.36	1.05 ± 0.21	-0.93 (-0.33 to -1.52)	1.08	0.01
Triglyceride (mg.dl ⁻¹)	51.7 ± 11.1	41.0 ± 8.9	-10.63 (- 21.26 to +0.00)	0.69	0.056
Cholesteryl ester (mg.dl ⁻¹)	7.7 ± 1.2	4.8 ± 3.0	-2.89 (-5.30 to -0.47)	0.83	0.03
Free cholesterol (mg.dl ⁻¹)	4.0 ± 0.9	2.6 ± 0.6	-1.45 (-2.51 to -0.39)	0.95	0.02
Phospholipid (mg.dl ⁻¹)	12.8 ± 3.0	9.2 ± 1.9	-3.55 (-7.48 to -0.38)	0.63	0.08
Protein (mg.dl ⁻¹)	8.0 ± 1.6	6.1 ± 1.1	-1.91 (-4.37 to -0.55)	0.54	0.12
TG/apoB ratio (mol:mol)	17751 ± 2507	24693 ± 4238	6942 (-1097 to +14982)	0.60	0.09
CE/TG ratio (mol:mol)	0.23 ± 0.03	0.17 ± 0.02	-0.06 (-0.15 to +0.03)	0.48	0.16

Values are mean ± SEM, n = 10.

Table 4.5; Concentration and composition of VLDL₁ in the postprandial states (240 minutes).

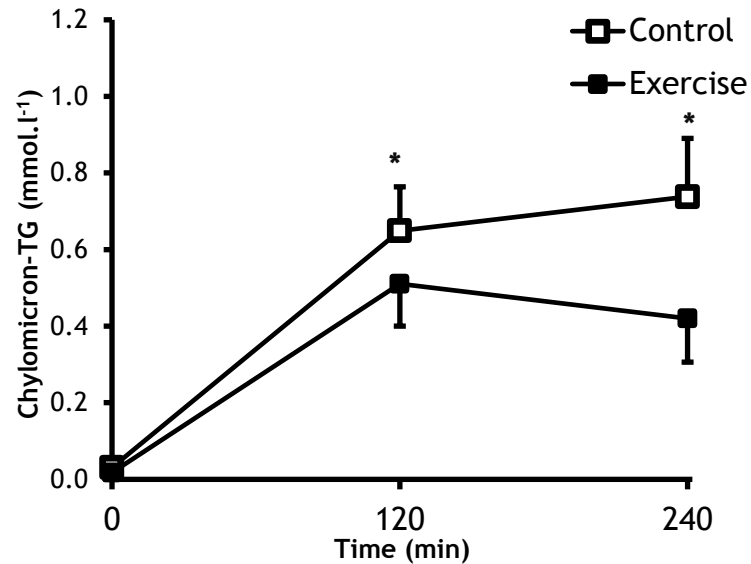
	Postprandial (240 minutes)				
	Control	Exercise	Different between trials and 95 % CI	Cohen's <i>d</i> effect	p-value
Total lipoprotein concentration (mg.dl ⁻¹)	137.9 ± 22.1	114.6 ± 18.0	-23.34 (-45.83 to -0.86)	0.72	0.049
ApoB (mg.dl ⁻¹)	2.22 ± 0.34	2.01 ± 0.31	-0.22 (-0.68 to -0.24)	0.33	0.33
Triglyceride (mg.dl ⁻¹)	88.5 ± 14.0	73.9 ± 12.3	-14.62 (-27.40 to -1.84)	0.79	0.03
Cholesteryl ester (mg.dl ⁻¹)	11.6 ± 2.0	8.3 ± 1.2	-1.33 (-2.59 to -0.07)	0.73	0.048
Free cholesterol (mg.dl ⁻¹)	5.9 ± 1.1	4.6 ± 0.8	-0.04 (-0.07 to -0.01)	0.92	0.045
Phospholipid (mg.dl ⁻¹)	21.1 ± 3.7	17.6 ± 8.7	+6.84 (0.28 to +9.77)	0.73	0.13
Protein (mg.dl ⁻¹)	10.8 ± 2.2	10.2 ± 1.2	-0.57 (-4.01 to +2.88)	0.11	0.73
TG/apoB ratio (mol:mol)	25103 ± 2304	23072 ± 2561	-2031 (-7969 to +3846)	0.24	0.47
CE/TG ratio (mol:mol)	0.18 ± 0.02	0.16 ± 0.01	-0.02 (-0.07 to +0.03)	0.28	0.40

Values are mean ± SEM, n = 10.

4 Effect of Exercise on the Affinity of Lipoproteins for Lipoprotein Lipase

[A] Time-averaged postprandial mean for chylomicron-TG concentrations

[B] Time-averaged postprandial chylomicron-TG concentrations



Time	0 min	120 min	240 min
Mean difference	0.01 ± 0.01	0.13* ± 0.08	0.24* ± 0.10

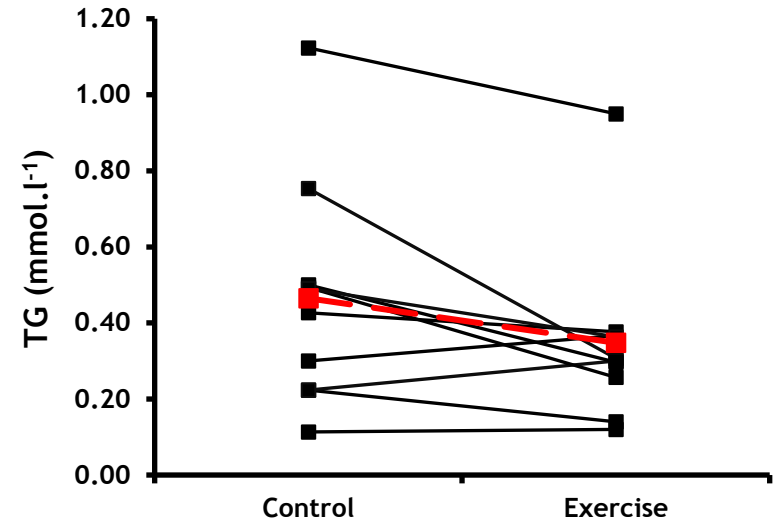
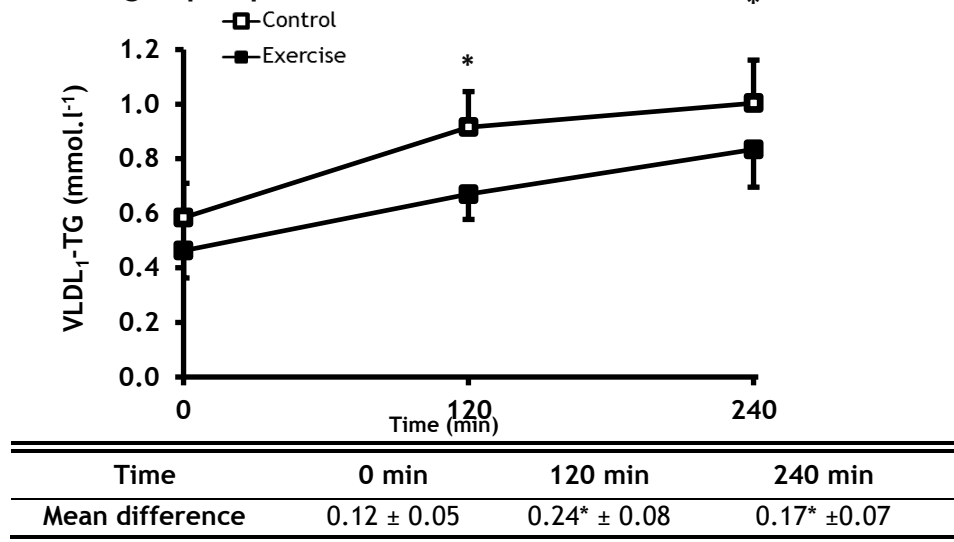
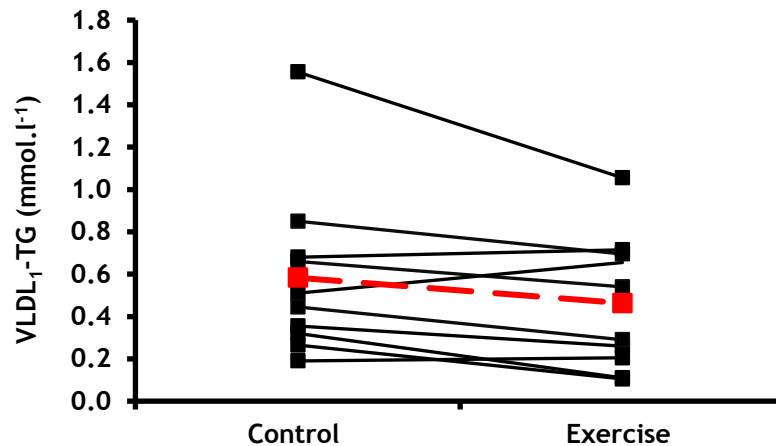


Figure 4.9. Values for chylomicron-TG in the Control and Exercise trials, panel [A] shows mean values for time-averaged postprandial chylomicron-TG concentrations, panel [B] shows individual values for time-averaged postprandial chylomicron-TG concentrations. Statistical analysis of these data is shown in Table 4.4 and 4.5. N = 10, Values are mean ± SEM. Black symbols show individual values, red symbols show the mean values. Values and statistical analysis of these data is shown in Table 4.2 and Table 4.3. * significant values from the other group (p = 0.005). The table below, shows the mean ± SEM differences between the two trials at each time point.* significant difference between trials at this time-point within subject (p < 0.005).

[A] Time-averaged postprandial mean for VLDL₁-TG concentrations*

[B] Fasted VLDL1-TG concentrations



[C] Time-averaged postprandial VLDL1-TG concentrations

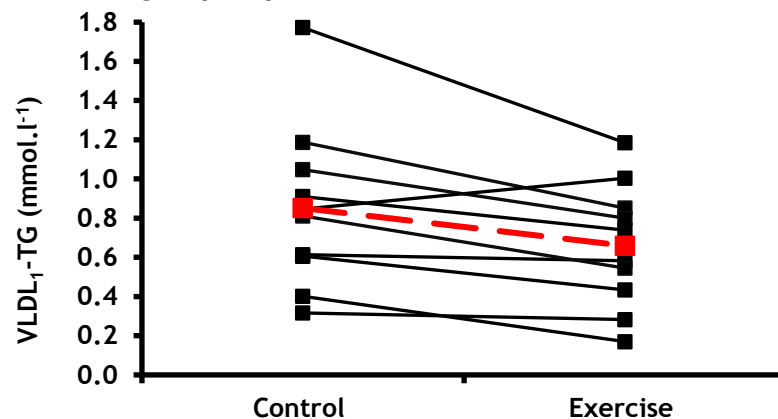


Figure 4.10. Values for VLDL₁-TG in the Control and Exercise trials, panel [A] shows mean values for time-averaged postprandial VLDL₁-TG concentrations, panel [B] shows individual values for fasted VLDL₁-TG concentrations, [C] shows individual values for time-averaged postprandial VLDL₁-TG concentrations. Statistical analysis of these data is shown in Table 4.4 and 4.5. N = 10, Values are mean ± SEM. The SEM shows the variability among the whole population. * significant difference between trials at this time-point within subject (p < 0.005). Black symbols show individual values, red symbols show the mean values. Values and statistical analysis of these data is shown in Table 4.2 and Table 4.3. * significant values from the other group (p = 0.005). The table below, shows the mean ± SEM differences between the two trials at each time point. * significant difference between trials at this time-point within subject (p < 0.005).

Table 4.6; Concentration and composition of VLDL₂ in the fasted state.

	Fasting (0 minutes)				
	Control	Exercise	Mean difference and 95% CI	Cohen's <i>d</i> effect size	p-value
Total lipoprotein concentration (mg.dl ⁻¹)	49.5 ± 3.6	39.4 ± 2.2	-10.08 (-24.20 to +4.04)	0.49	0.15
ApoB (mg.dl ⁻¹)	4.00 ± 0.57	3.39 ± 0.17	-0.61 (- 2.52 to +1.30)	0.22	0.50
Triglyceride (mg.dl ⁻¹)	18.2 ± 3.2	15.0 ± 2.5	-3.21 (-8 .84 to +2.42)	0.35	0.29
Cholesteryl ester (mg.dl ⁻¹)	11.3 ± 3.0	9.1 ± 1.5	-2.16 (-7.43 to +3.12)	0.28	0.39
Free cholesterol (mg.dl ⁻¹)	4.0 ± 0.8	3.3 ± 0.5	0.38 (-1.18 to +1.93)	0.17	0.22
Phospholipid (mg.dl ⁻¹)	9.8 ± 0.9	8.3 ± 0.5	1.55 (-5.06 to +1.96)	0.31	0.36
Protein (mg.dl ⁻¹)	6.1 ± 0.4	3.7 ± 0.3	-2.43 (-5.11 to +0.26)	0.63	0.08
TG/apoB ratio (mol:mol)	3679 ± 567	3051 ± 400	-628 (-1600 to +343)	0.45	0.19
CE/TG ratio (mol:mol)	0.59 ± 0.10	0.62 ± 0.06	0.03 (-0.24 to +0.30)	0.09	0.79

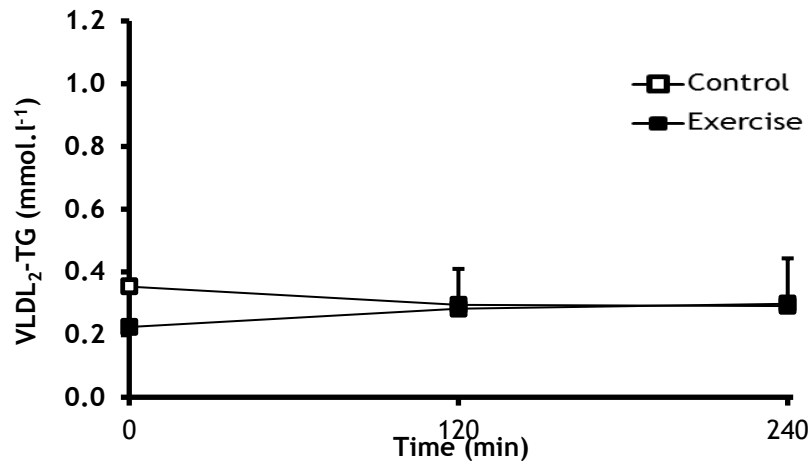
Values are mean ± SEM, n = 10. *Statistical analysis performed on log-transformed data, and values are geometric mean (95% confidence interval).

Table 4.7; Concentration and composition of VLDL₂ in the postprandial state.

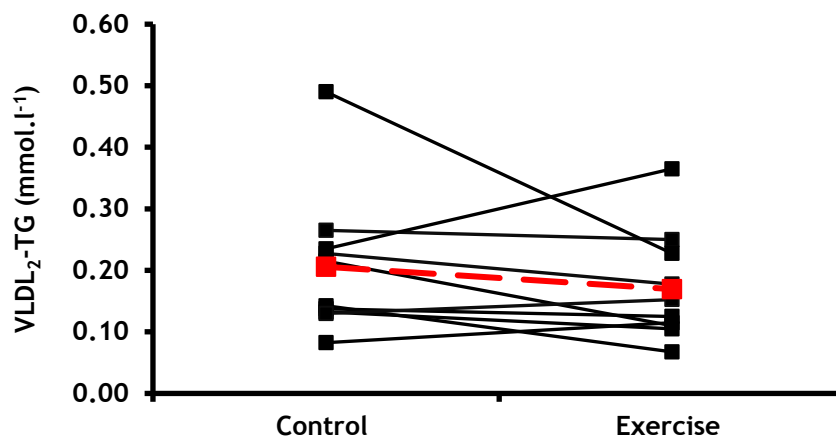
	Postprandial (240 minutes)				
	Control	Exercise	Different between trials and 95 % CI	Cohen's <i>d</i> effect size	p-value
Total lipoprotein concentration (mg.dl ⁻¹)	38.2 ± 1.6	40.0 ± 2.8	+1.80 (-5.35 to +8.93)	0.38	0.59
ApoB (mg.dl ⁻¹)	2.43 ± 0.17	2.53 ± 0.15	+0.10 (-0.32 to +0.53)	0.37	0.60
Triglyceride (mg.dl ⁻¹)	15.8 ± 2.2	16.2 ± 2.3	+0.44 (-3.98 to +4.86)	0.07	0.83
Cholesteryl ester (mg.dl ⁻¹)	7.2 ± 1.5	6.7 ± 1.2	-0.44 (-1.63 to +0.76)	0.43	0.44
Free cholesterol (mg.dl ⁻¹)	3.0 ± 0.5	2.9 ± 0.4	-0.12 (-0.54 to 0.30)	0.57	0.56
Phospholipid (mg.dl ⁻¹)	7.3 ± 0.5	7.6 ± 0.6	+0.33 (-1.48 to +2.14)	0.13	0.70
Protein (mg.dl ⁻¹)	5.0 ± 0.2	6.6 ± 0.7	+1.59 (-1.07 to +4.24)	0.17	0.22
TG/apoB ratio (mol:mol)	5416 ± 993	4169 ± 540	900592 (-609330 to +790513)	0.09	0.20
CE/TG ratio (mol:mol)	0.45 ± 0.08	0.43 ± 0.04	-28.34 (-89.91 to +33.22)	0.32	0.78

Values are mean ± SEM, n = 10. *Statistical analysis performed on log-transformed data, and values are geometric mean (95% confidence interval).

[A] Mean Time-averaged postprandial mean for VLDL₂- TG concentrations



[B] Fasted VLDL₂-TG concentrations



[C] Time-averaged postprandial VLDL₂-TG concentrations

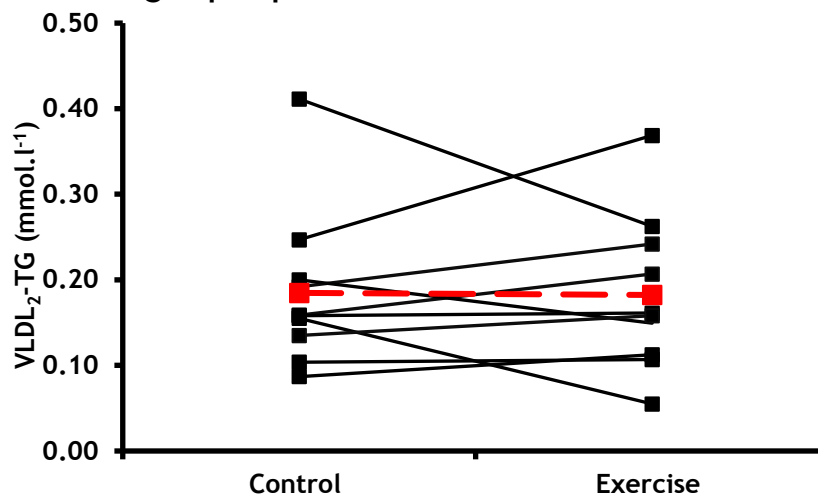


Figure 4.11. Values for VLDL₂-TG in the Control and Exercise trials, panel [A] shows mean values for time-averaged postprandial VLDL₂-TG concentrations, panel [B] shows individual values for fasted VLDL₂-TG concentrations [C] shows individual values for time-averaged postprandial VLDL₂-TG concentrations. Statistical analysis of these data is shown in Table 4.4 and 4.5. N = 10, Values are mean \pm SEM. Black symbols show individual values, red symbols show the mean values. Values and statistical analysis of these data is shown in Table 4.2 and Table 4.3. * significant values from the other group ($p = 0.005$).

4.3.3 Lipoprotein affinity for LPL

Figure 4.12, panel A, Figure 4.13, panel A, and Figure 4.14, panel A show mean (\pm SEM) NEFA release over the 30 minute incubation period in the LPL-affinity assay for VLDL₁ and VLDL₂ in the fasted state and 240 minutes postprandially, and for chylomicrons 240 minutes postprandially in the control and exercise trials. Affinity of chylomicrons, VLDL₁ and VLDL₂ for LPL was calculated from the gradient of the linear portion (before a plateau was achieved) of the individual NEFA release versus time plots. Table 4.8 shows VLDL₁ and VLDL₂ affinity for LPL in the fasted state and 240 minutes postprandially and for chylomicrons 240 minutes postprandially, expressed as mmol of NEFA release, per mmol lipoprotein-TG, per unit LPL activity, per minute. Exercise did not affect the affinity of postprandial chylomicron, there was substantial individual variability in the exercise effect (6 out of 10 increased with exercise in postprandial state), leading to non-significant effects of exercise on both fasting (mean (95%CI) difference: (0.20 (-0.39 to 0.78) mmol of NEFA release, per mmol lipoprotein TG, per unit LPL activity, per minute (Figure 4.12, panel B). The Cohen's d effect sizes at 0.21, for the change in chylomicron affinity with exercise were smaller than the effect of exercise on VLDL₁ affinity. The exercise significantly increased the affinity of VLDL₁ for LPL in 8 out of 10 and 9 out of 10 (Figure 4.13, panel C and D) in both fasted and postprandial states, (0.98 (0.55 to 1.41); $p=0.01$) and (1.16 (0.63 to 1.70); $p<0.001$), respectively, with a large Cohen's d effect size of 1.58 and 1.35 respectively. Exercise did not affect neither fasted nor postprandial VLDL₂ affinity for LPL, with mean difference of (0.01 (-0.17 to 1.64); $p=0.60$) and (0.25 (0.12 to 1.45); $p=0.34$) (Figure 4.14 panel C and D). The Cohen effect size was small in both fasted and postprandial states 0.13 and 0.34 respectively.

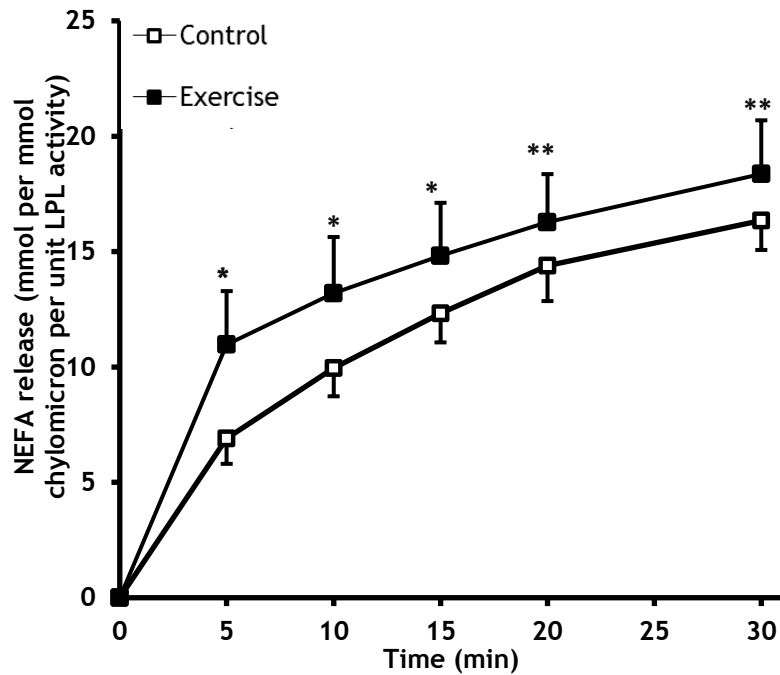
Exercise increased affinity of VLDL₁ for LPL by 2.2 (1.3 to 3.7) fold (geometric mean (95% CI)) in the fasted state (p= 0.02 for fold increase; p value for absolute increase shown in **Table 4.8**) and by 2.6 (1.8 to 3.8) fold in the postprandial state (p=0.001 for fold increase; p value for absolute increase shown in **Table 4.8**). However, there was no significant change in affinity of chylomicrons for LPL in either fold (1.2 (0.6 to 2.3) fold; p=0.59) or absolute units (see **Table 4.8**). Affinity of VLDL₂ for LPL was negligible, ~50-100-fold lower than chylomicrons and zero in many instances, and did not change in response to exercise. In the control trial, the affinity of chylomicrons for LPL was 11.3 (6.0 to 21.6) fold greater (p=0.0001) than the affinity of VLDL₁ in the postprandial state, whereas in the exercise trial affinity of chylomicrons for LPL was 6.0 (3.0 to 12.0) greater (p=0.0007) than the affinity of VLDL₁ in the postprandial state. Thus, the affinity of postprandial VLDL₁ for LPL-mediated TG-hydrolysis relative to the affinity of chylomicrons was 2.6 (1.3 to 5.4) fold greater in the exercise compared to the control trial (p=0.03). There was no significant difference in affinity of VLDL₁ for LPL between the fasted and postprandial states in either the control (postprandial VLDL₁ LPL affinity 0.7 (0.4 to 1.1) fold compared with fasting VLDL₁ LPL affinity, p=0.18) or the exercise (postprandial VLDL₁ LPL affinity 0.8 (0.6 to 1.1) fold compared with fasting VLDL₁ LPL affinity, p=0.28) trials.

Table 4.8; Lipoprotein Affinity for LPL.

		NEFA release (mmol.min ⁻¹ per mmol lipoprotein-TG per unit LPL activity)			
		Control	Exercise	Cohen d effect size	p-value
Fasting (0 minutes)	VLDL ₁	0.16 (0.09 to 0.29)	0.35 (0.24 to 0.52)	1.58	0.018*
	VLDL ₂	0.013 (0.004 to 0.044)	0.018 (0.007 to 0.049)	0.13	0.60
	CM**	1.25 (0.94 to 1.66)	1.52 (0.98 to 2.34)	0.21	0.53*
Postprandial (240 minutes)	VLDL ₁	0.08 (0.03 to 0.19)	0.25 (0.15 to 0.42)	1.35	0.002*
	VLDL ₂	0.021 (0.006 to 0.070)	0.013 (0.004 to 0.048)	0.34	0.34

Values are geometric mean (95% confidence interval), n = 10. *Statistical analysis and mean differences performed on log-transformed data.** (CM) chylomicron.

[A] Mean values for NEFA release from chylomicron in postprandial state



[B] Individual values for NEFA release from chylomicron in postprandial state

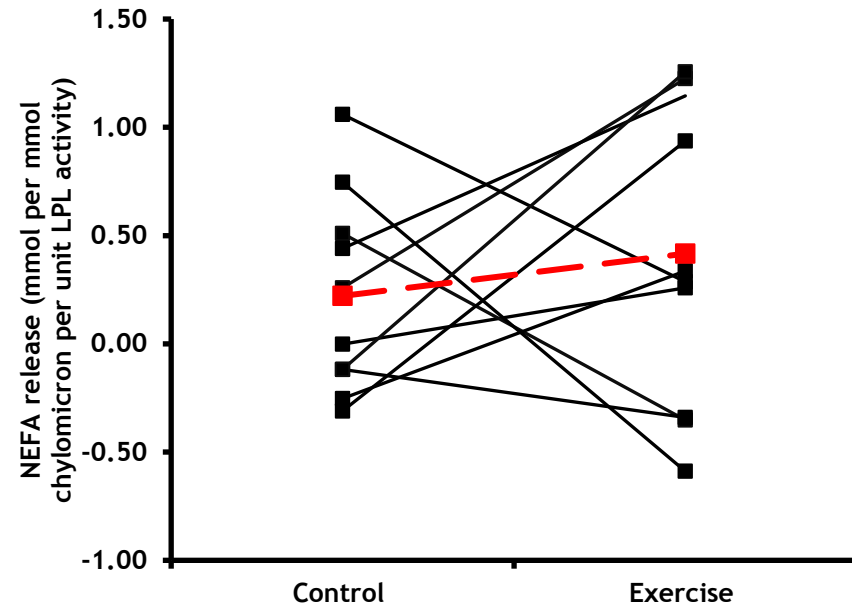
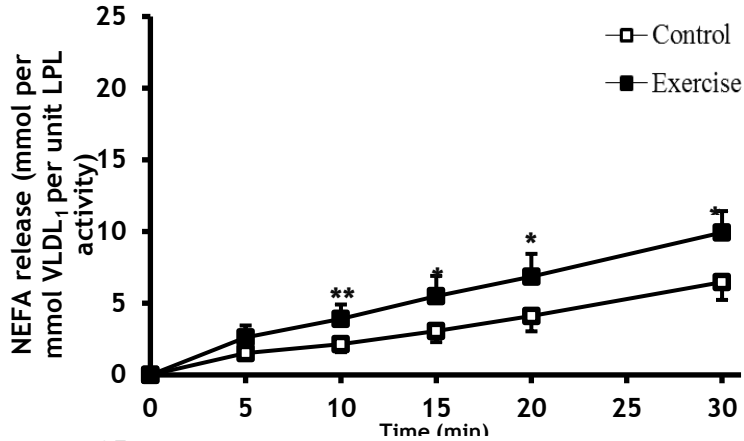


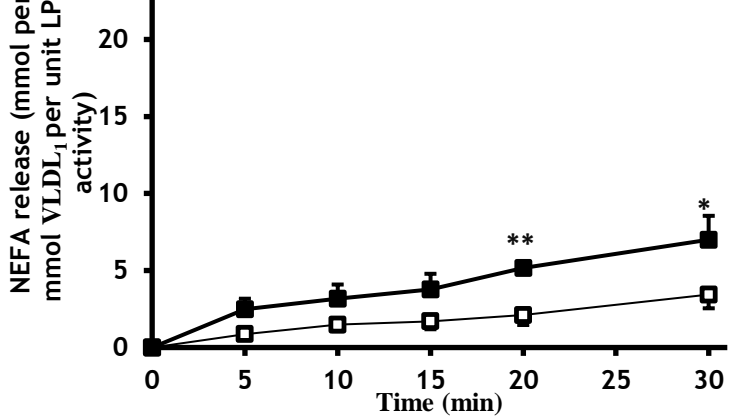
Figure 4.12. Values for NEFA release over 30-minutes in LPL-affinity assay in chylomicron in postprandial state Control and Exercise trials, panel [A] shows mean value of NEFA release over 30-minutes from chylomicron, panel [B] shows the individual value of NEFA release over 30-minutes from chylomicron. N = 10, Values are mean \pm SEM. The SEM shows the variability among the whole population. Affinity of lipoproteins for LPL was determined by the rate of NEFA release over the linear portion of the 30-minute incubation period before a plateau was achieved for each individual participant. Values for lipoprotein affinity for LPL and statistical analyses are shown in Table 4.8. Significant values from the other group * ($p = 0.005$) and ** ($p < 0.05$).

4 Effect of Exercise on the Affinity of Lipoproteins for Lipoprotein Lipase

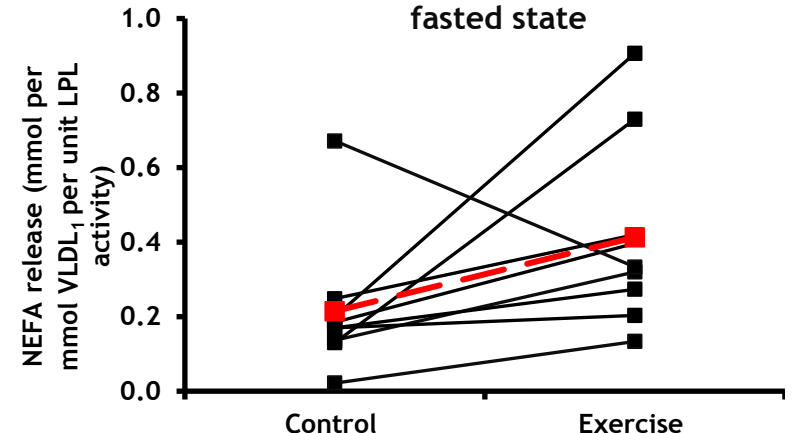
[A] Mean values for NEFA release from VLDL₁ in fasted state



[B] Mean values for NEFA release from VLDL₁ in postprandial state



[C] Individual values for NEFA release from VLDL₁ in fasted state



[D] Individual values for NEFA release from VLDL₁ in postprandial state

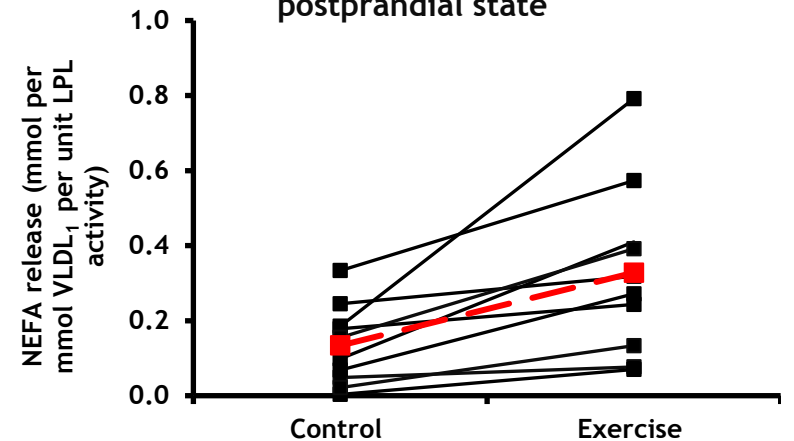


Figure 4.13. Values for NEFA release over 30-minutes in LPL-affinity assay in VLDL₁ in fasted and postprandial state in Control and Exercise trials, panel [A] shows mean value of NEFA release over 30-minutes from VLDL₁ in fasted state, panel [B] shows mean value of NEFA release over 30-minutes from VLDL₁ in postprandial state, panel [C] shows the Individual value of NEFA release over 30-minutes from VLDL₁ in fasted state, panel, panel [D] shows the Individual value of NEFA release over 30-minutes from VLDL₁ in fasted state. N = 10, Values are mean \pm SEM. The SEM shows the variability among the whole population. Affinity of lipoproteins for LPL was determined by the rate of NEFA release over the linear portion of the 30-minute incubation period before a plateau was achieved for each individual participant. Values for lipoprotein affinity for LPL and statistical analyses are shown in Table 4.8 Significant values from the other group * ($p = 0.005$) and ** ($p < 0.001$).

4 Effect of Exercise on the Affinity of Lipoproteins for Lipoprotein Lipase

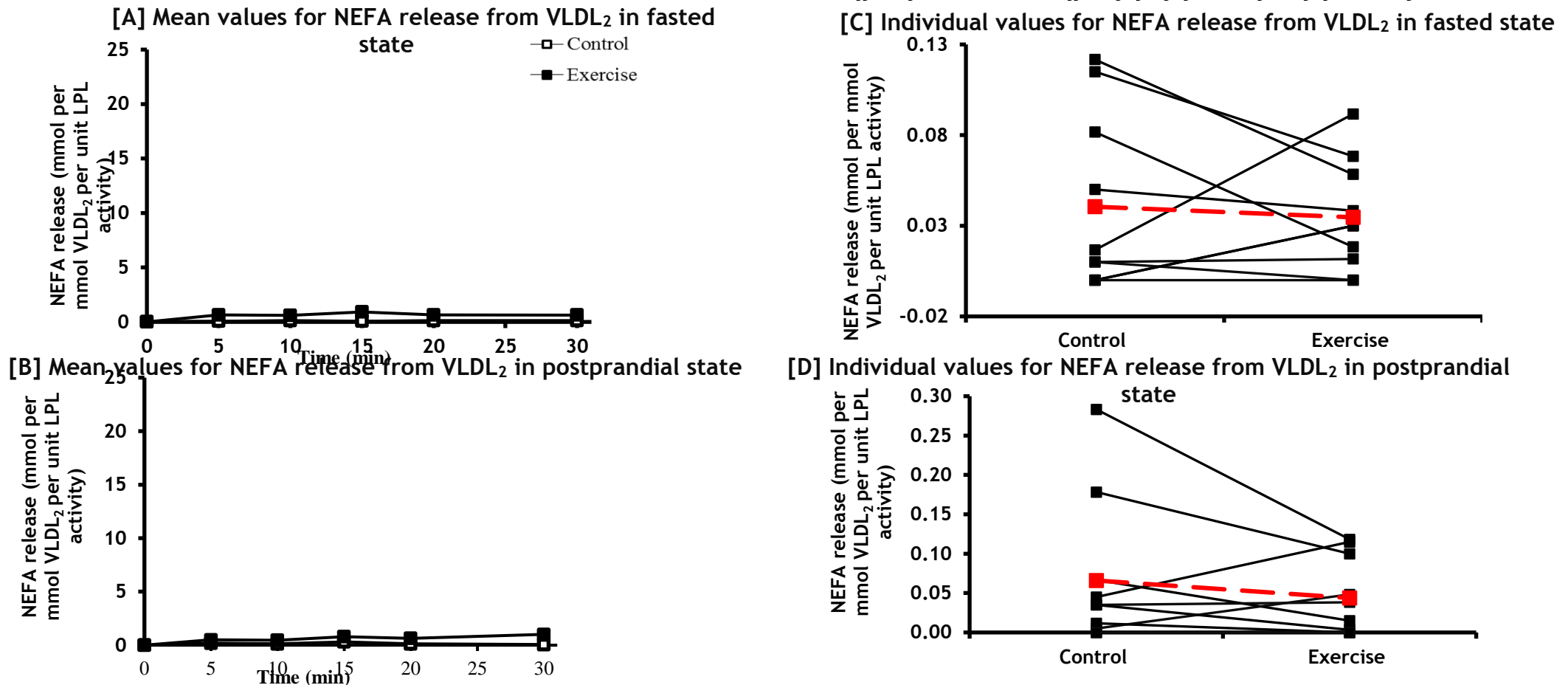


Figure 4.14. Values for NEFA release over 30-minutes in LPL-affinity assay in VLDL₂ in fasted and postprandial state in Control and Exercise trials, panel [A] shows mean value of NEFA release over 30-minutes from VLDL₂ in fasted state, panel [B] shows mean value of NEFA release over 30-minutes from VLDL₂ in postprandial state, panel [C] shows the individual value of NEFA release over 30-minutes from VLDL₂ in fasted state, panel, panel [D] shows the individual value of NEFA release over 30-minutes from VLDL₂ in fasted state. N = 10, Values are mean \pm SEM . The SEM shows the variability among the whole population. Affinity of lipoproteins for LPL was determined by the rate of NEFA release over the linear portion of the 30-minute incubation period before a plateau was achieved for each individual participant. Values for lipoprotein affinity for LPL and statistical analyses are shown in Table 4.8. Significant values from the other group * (p = 0.005) and ** (p < 0.05).

4.4 Discussion

Elevated postprandial TG concentrations are associated with increased risk of cardiovascular events (Bansal et al., 2007, Nordestgaard and Nielsen, 1994, Mora et al., 2008), and there is evidence that this relationship is likely to be causal (Goldberg IJ, 2011, Chapman et al., 2011, Jørgensen et al., 2012, Consortium and Collaboration, 2010, Cohorts, 2014, Jørgensen et al., 2014). Prior exercise has consistently been shown to lower postprandial TG concentrations, but the mechanisms responsible have been unclear (Gill and Hardman, 2000, Gill et al., 2004a, Farah and Gill, 2012). Previous studies have shown that TG reductions in large VLDL₁ make the largest quantitative contribution to the overall TG-lowering effect of exercise (Gill et al., 2006); that exercise lowers VLDL₁-TG concentrations by increasing clearance of these lipoproteins from the circulation, rather than reducing their production (Al-Shayji et al., 2012); but that exercise-induced TG-lowering is often observed in the absence of an elevation in post-heparin plasma or skeletal muscle LPL activity (Malkova and Gill, 2006, Harrison et al., 2012). The main novel finding of the present study is that prior exercise increased the affinity of large VLDL₁ - but not of chylomicrons or VLDL₂ - for clearance by LPL. This provides an important advance in our understanding of the mechanism by exercise lowers TG concentrations; an effect which is likely to contribute to exercise's overall cardioprotective benefit.

The present findings put previous observations about the effects of exercise TG-rich lipoprotein metabolism into context. Earlier work demonstrated that exercise often lowered TG concentrations without a substantial increase in post-heparin plasma or skeletal muscle LPL activity (Harrison et al., 2012, Malkova and Gill, 2006, Gill et al., 2006, Herd et al., 2001); that the TG reductions induced by exercise are typically larger in VLDL than chylomicrons (Malkova and Gill, 2006, Gill and Hardman, 2000, Gill et al., 2006); and that exercise increased clearance of VLDL from the circulation but did not reduce hepatic VLDL production (Tsekouras et al., 2007, Magkos et al., 2006).

However, these observations could not explain how this increased VLDL clearance could occur without a concomitant increase in LPL activity. More recently, we demonstrated that the exercise-induced increase in clearance of VLDL₁-TG was twice as large as the exercise-induced increase in clearance of TG from

chylomicron-like particles (Al-Shayji et al., 2012). This suggested that exercise upregulated clearance of VLDL₁ to a greater extent than chylomicrons, which led to the hypothesis that exercise increased the affinity of VLDL₁ for TG-hydrolysis by LPL. This hypothesis was confirmed by the findings of the present study. Exercise increased the affinity of VLDL₁ for LPL clearance by 2.2-fold in the fasted state ($p = 0.02$) and 2.6-fold in the postprandial state ($p = 0.001$), but did not significantly alter the affinity of chylomicrons for LPL-mediated TG-hydrolysis. Accordingly the affinity of VLDL₁ relative to chylomicrons for LPL-mediated clearance in the postprandial state was 2.6-fold greater in the exercise compared with the control trial ($p = 0.03$). Data shown there was a numerical increase in chylomicron affinity for LPL of 1.2 fold. However there was a large amount of individual variability in this effect - six of the 10 had an increase in chylomicron affinity for LPL in response to exercise, the other four subjects experienced a decrease, which meant that the 95% confidence interval around this point was large (0.6-2.3 fold) and the p -value for the fold change was 0.59 ($p=0.53$ for absolute change in affinity). The Cohen's d for the effect of exercise on chylomicron affinity for LPL was 0.21, which is considered a small effect size, whereas the effect size for the effect of exercise on VLDL₁ affinity for LPL was 1.58 in the fasted state and 1.35 in the postprandial state, which are both considered to be large effect sizes (Cohen, 2013). This magnitude of this change in relative VLDL₁ affinity for LPL-mediated TG-hydrolysis is consistent our earlier observation that the exercise-induced increase in VLDL₁-TG clearance was twice as great as the exercise-induced increase in chylomicron-like particles (Al-Shayji et al., 2012).

It is not clear from the present findings how exercise increases the affinity of VLDL₁ for LPL-mediated TG hydrolysis. We previously demonstrated that following exercise, VLDL₁ particles were larger (higher TG/apoB ratio) and more TG-enriched (lower CE/TG ratio) and that exercise-induced increases the fractional catabolic rate of VLDL₁ particles were correlated with the increases in TG-enrichment and size of the VLDL₁ particle (Al-Shayji et al., 2012). As larger, more TG-enriched lipoprotein particles have greater affinity for LPL (Saheki et al., 1991, Fisher et al., 1995), we hypothesised that this compositional change was a potential mediator of increased affinity of VLDL₁ for LPL post-exercise (Al-Shayji et al., 2012). In the Al-Shayji study, VLDL₁ composition was only measured in the fasted state, and in the present study we observed changes of similar magnitude

in the TG/apoB ratio (39% increase with exercise in present study vs 26% increase in Al-Shayji et al and CE/TG ratio (26% decrease with exercise in present study vs 29% decrease in Al-Shayji et al in fasting VLDL₁ composition, although these compositional changes did not quite achieve statistical significance in the present study (Al-Shayji et al., 2012).

This would be consistent with this compositional change contributing to the enhanced affinity for LPL-mediated clearance. However, in the postprandial state the VLDL₁ TG/apoB ratio and CE/TG ratio were similar in the exercise and control, despite the affinity of VLDL₁ for LPL-mediated TG hydrolysis in response to exercise increasing to a similar extent in the postprandial state to that observed in the fasted state. The similar postprandial VLDL₁ CE/TG ratio observed in the control and exercise trials also contrasts with an earlier observation that the VLDL₁ CE/TG ratio was lower following exercise postprandially (Gill et al., 2006). This may indicate that the increase in VLDL₁ affinity for LPL in response to exercise was mediated by factors other than changes in size and TG-enrichment of the VLDL₁ particle. However, there are two other aspects worth consideration when interpreting these findings. First, it is important to recognise that all circulating VLDL₁ particles are essentially remnant particles to some extent, in that they will have had a degree of LPL-mediated hydrolysis of their TG-core by the time the blood sample was taken. Post-exercise VLDL₁ particles with greater affinity for LPL would have been exposed to proportionately greater TG hydrolysis by the time a blood sample was taken. Thus, relative to newly secreted VLDL₁ particles, circulating post-exercise VLDL₁ particles may have had their size and TG-enrichment reduced to a greater extent than VLDL₁ particles in the control trial. Thus, it is possible that following exercise, the liver produced larger, more TG-enriched VLDL₁ particles and that this contributed to their increased affinity for VLDL₁, but this effect was not fully reflected in the composition of the measured circulating VLDL₁ particle. However, in Al-Shayji *et al*, ratio of VLDL₁-TG production to VLDL₁-apoB production was similar in control and exercise trials, suggesting that the average size of the secreted VLDL₁ particle was not influenced by exercise (Al-Shayji et al., 2012). However, it is also important to recognise that VLDL₁ particles are heterogeneous, occupying a greater than three-fold range in size and density, and thus considering only 'average' lipoprotein size and composition in the VLDL₁ range may not reveal the whole story. Interestingly, Al-

Shayji *et al* reported a larger increase in the VLDL₁-apoB fractional catabolic rate than the VLDL₁-TG fractional catabolic rate with exercise (146% vs 82% increase) (Al-Shayji *et al.*, 2012), which would suggest that exercise was having a proportionately larger effect on clearance of smaller, less TG-rich VLDL₁ particles (which have a lower TG/apoB ratio), either via direct particle removal or by TG-removal taking them out of the VLDL₁ and into the VLDL₂ density range. This interpretation would suggest that the observation that circulating VLDL₁ particles post-exercise are larger and more TG-enriched reflects the fact that exercise disproportionately increased clearance of smaller, less TG-enriched particles at the lower end of the Sf 60-400 range, leaving proportionally more larger VLDL₁ in the circulation. Accordingly, it is possible that the strong correlations between change in VLDL₁ apoB fractional catabolic rate and change in VLDL₁ TG/apoB and CE/TG ratio observed in that study (Al-Shayji *et al.*, 2012) reflects greater clearance of smaller VLDL₁ particles following exercise, which would have the net effect of increasing the average TG/apoB and CE/TG ratio of the remaining circulating lipoprotein particles across the VLDL₁ density range. This interpretation is supported by a recent study by Harrison and colleagues who used nuclear magnetic resonance spectroscopy to quantify 24 different VLDL subfractions in the fasted state following exercise (Harrison *et al.*, 2012). This report found that in response to exercise, there was a proportionally larger TG reduction in 'medium VLDL' (size range 43-55 nm, approximately corresponding to Sf 100-200 (Redgrave, 2004), i.e. smaller VLDL₁) than in 'large VLDL' (size (55-260 nm, approximately corresponding to Sf >200 (Redgrave, 2004), i.e. larger VLDL₁), with VLDL particles over 120 nm in size being virtually unaffected by exercise (Harrison *et al.*, 2012). The authors proposed that their findings were suggestive of independent metabolic regulation of different VLDL pools within the VLDL₁ range (Harrison *et al.*, 2012). This is an attractive proposition which is consistent with our present and earlier (Al-Shayji *et al.*, 2012) observations on the effects of exercise on VLDL₁ metabolism. Thus, further study is needed to both examine the effects of exercise on the affinity of smaller and larger VLDL₁ particles for LPL-mediated TG hydrolysis and to understand the effects of exercise on TG and apoB kinetics of smaller and larger VLDL₁ particles.

While the present findings clearly show that exercise increased the affinity of VLDL₁ particles for clearance by LPL, this effect is unlikely to be solely responsible

for the observed TG lowering effect of exercise. We observed a significant reduction in chylomicron-TG concentration following exercise, without a corresponding increase in the affinity for chylomicrons for LPL-mediated clearance. This chylomicron-TG reduction is consistent with earlier observations (Gill et al., 2006, Gill and Hardman, 2000) and is likely to reflect increased LPL-mediated chylomicron TG clearance, in line with our recent report that Intralipid-TG fractional catabolic rate was increased by prior exercise (Al-Shayji et al., 2012). We did not measure LPL activity in the present study, so it is unclear whether this is the consequence of increased LPL activity or exercise-induced changes in blood perfusion to LPL-rich tissues leading to increased interactions between chylomicron particles and LPL, or a combination of the two. It has previously been reported that postprandial blood flow to the leg was almost 40% higher on the day following 2 hours of walking (Malkova et al., 2000), so it is conceivable that the reduction in chylomicron-TG observed in the present study could have occurred without a marked increase in LPL activity, which would be consistent with previous studies which have generally reported that LPL activity is not substantially elevated in response to exercise of the nature undertaken here (Harrison et al., 2012, Malkova and Gill, 2006, Herd et al., 2001). It is a limitation to the study that we did not measure LPL activity. This information would have provided a more complete assessment of the relative importance of increased VLDL₁ affinity for LPL vs increased LPL activity in mediating the overall TG-lowering effect of exercise. However, the invasive muscle biopsies required to determine skeletal muscle LPL activity were not feasible, and because heparin injection distorts lipoprotein metabolism, it would not have been possible to obtain fasting and postprandial post-heparin plasma LPL activity assessments in parallel with other measurements made in the study. Nevertheless, it would be helpful if future studies investigating the effects of exercise on kinetics and affinity for LPL of VLDL sub-populations, could also directly measure LPL activity.

A further observation was that exercise substantially reduced fasting sdLDL cholesterol concentrations. There is accumulating evidence that sdLDL have particularly high atherogenicity (Hirayama and Miida, 2012, Diffenderfer and Schaefer, 2014), thus, exercise-induced reductions are likely to have clinically relevant implications for CVD risk. Elevated concentrations of TG-rich lipoproteins facilitate the development of sdLDL by accelerating CETP-mediated neutral lipid

exchange, between TG-rich lipoproteins and LDL, leading to TG-enriched LDL particles, which are then acted on by hepatic lipase to produce sdLDL particles (Hirayama and Miida, 2012, Diffenderfer and Schaefer, 2014). Thus, by lowering TG-rich lipoprotein concentrations, exercise is likely to have inhibited the neutral lipid exchange process leading to sdLDL formation.

It is interesting TG-lowering effect of exercise occurs in absence of significant change of insulin and glucose concentrations. It was established that insulin resistance is an important determinant of TGR lipoproteins metabolism. However, in this instant TG-lowering effect may be independent of insulin sensitivity; this is consistent with earlier reports that TG-lowering effect is independent from insulin (Gill et al., 2002).

In conclusion, this study's main finding that exercise increases the affinity of VLDL₁ for LPL-mediated TG-hydrolysis provides an important piece in the jigsaw of understanding the effects of exercise on the metabolism of TG-rich lipoproteins. These data provide clarity to the interpretation of the findings of many studies into the effects of exercise on postprandial lipoprotein undertaken over the past 15-20 years, explaining why exercise often affects VLDL₁-TG concentrations to a greater extent than chylomicron concentrations, and why a TG-lowering effect of exercise is often seen without an increase in LPL activity. However, it is still unclear how exercise increases the affinity of VLDL₁ for LPL-mediated clearance and further study is now needed to understand the effects of exercise on the affinity for LPL and kinetics of sub-populations of VLDL₁.

5 Effect of co-ingesting fat with carbohydrate on lipid and glucose response

5.1 Introduction

In response to meal ingestion, there are a number of perturbations to metabolism which are likely to impair insulin sensitivity and possibly contribute to atherosclerotic progression. As humans spend most of their time in the postprandial state, these metabolic changes have possible relevance in the development of the metabolic syndrome and T2D, and also increase CVD risk. There are a number of plausible mechanisms by which this can occur. Ingestion of fat leads to increases in postprandial chylomicron and VLDL concentrations. The magnitude of these changes is associated with the amount (Schwab et al., 2014) and type (saturated, monounsaturated, polyunsaturated) of fat consumed. These postprandial lipoproteins and their remnants may deposit into arterial walls accelerating the development of atheromatous plaques (Zilversmit, 1979). In addition, high concentrations of these triglyceride-rich lipoproteins facilitate neutral lipid exchange with HDL and LDL, mediated by CETP, contributing to the generation of an atherogenic lipoprotein phenotype (Zhong et al., 1996, Cohen et al., 1994).

Ingestion of carbohydrate induces a rise in blood glucose concentrations which triggers the release of insulin from the pancreas (Scheen, 2004, Del Prato and Tiengo, 2001, Wallum et al., 1992, Brand-Miller, 2004). There is evidence that these postprandial increases in glucose and insulin may have adverse effects on insulin sensitivity, diabetes risk and obesity (Giovannucci, 1995, Daly, 2003) and thus, minimising the extent of these postprandial perturbations may be beneficial. For example, it has been shown that consuming foods with a low glycaemic index can reduce insulin demand, improve blood glucose control, and reduce blood lipid levels, all factors that may play important roles in the prevention or management of metabolic disease (Augustin et al., 2002, Jenkins et al., 1990). The magnitude of these postprandial changes is related to the amount of carbohydrate consumed

(Blaak, 2016, Wolever and Bolognesi, 1996b, Wolever and Mehling, 2003, Sheard et al., 2004), but the type of carbohydrate ingested also plays a key role (Sheard et al., 2004, Salmerón et al., 1997). The glycaemic index (GI) quantifies the postprandial glucose rise for a given food, relative to that for glucose. Foods with lower GIs lead to lower glucose and insulin responses (Jenkins et al., 2008). In epidemiological studies low GI diets are associated with reduced risk of CVD and diabetes (Barclay et al., 2008, Qi and Hu, 2007, Livesey et al., 2008), lower plasma TG by around 15% and cholesterol by 8% (Brand-Miller et al., 2003, Pelkman, 2001). There are benefits of using low-GI diets in the management of diabetes (Brand-Miller et al., 2003, Livesey et al., 2008, Rahelić et al., 2011) and interventions with low GI diets (LGI) have shown a reduction in fasting plasma glucose and postprandial plasma glucose and insulin in type-2 diabetic by around 30% (Jenkins et al., 1988). The diets are reported to reduce the level of HbA_{1c} by absolute amounts varying from 3% (Calle-Pascual et al., 1987) to 19% (Jenkins et al., 1988, Augustin et al., 2002, Jenkins et al., 2008). Another study (Rizkalla et al., 2004) found that also, a low GI diet was able to improve glycaemic control, glucose utilization, some lipid profiles, and the capacity for fibrinolysis in type 2 diabetes (Rizkalla et al., 2004). In addition, a low-GI diet has been shown to improve the lipid profile (Thomas et al., 2007, Wolever et al., 1992). High GI diets, on the other hand, are associated with insulin resistance, type 2 diabetes, dyslipidaemia and prostate, breast and colon cancer (Blaak, 2016, Pi-Sunyer, 2002, McKeown-Eyssen, 1994, Giovannucci, 1999, Giovannucci, 1995).

In free-living conditions, humans generally co-ingest fat and carbohydrate, so it is important to consider the effect consuming fat with carbohydrate on postprandial glucose, insulin and NEFA responses compared with ingestion of carbohydrate alone, as it is possible that this may slow glucose release into the circulation, thereby lowering the meal's GI (Järvi et al., 1999, Wolever et al., 1999, Wolever et al., 1994, Moghaddam et al., 2006). Conversely, the addition of carbohydrate to fat ingestion will induce a larger insulin response (Moghaddam et al., 2006, Mohammed and Wolever, 2004), which would be expected to induce greater suppression of NEFA release from adipose tissue, a larger upregulation of adipose tissue LPL activity (Knuth et al., 2008, Knuth et al., 2007), and a greater suppression of hepatic VLDL

production (Schneeman et al., 1993), which might be expected to attenuate the postprandial TG response (Jeppesen et al., 1995). However, the effects of such coingestion on the concentration and composition different lipoprotein species in the postprandial state are not known.

Therefore, the aim of this experimental chapter is to investigate the acute effects of consuming fat alone, carbohydrate alone and co-ingestion of fat and carbohydrate on postprandial metabolic responses, particularly on the concentration and composition of postprandial lipoprotein species.

5.2 Participants and Methods

5.2.1 Participants

Thirteen participants were initially recruited but three withdrew before completion of the experimental period for undisclosed personal reasons. Thus 10 apparently healthy men not taking any drugs thought to affect lipid or carbohydrate metabolism took part in the study. Their demographic information is given in **Table 5.1** below.

Table 5.1; Demographic data.

Age (years)	30.0 ± 3.9
Weight (kg)	87.4 ± 10.9
Height (m)	182.6 ± 6.9
BMI (kg.m ⁻²)	26.3 ± 4.1
Waist/ hip ratio	0.86 ± 0.07
Percentage fat (%)	20.6 ± 6.9

5.2.2 Study design

A cross-over study design was used with participants undertaking three postprandial assessments: an OFTT (75 g of fat), an OGTT (75 g of Glucose) and combination of both (COMB) (75 g of fat and 75 g of glucose) in a randomised order with an interval of 7-14 days between tests. In each test, blood samples were collected in the fasted state and over 8 hours postprandially. **Figure 5.1** shows the experimental design, which is described in detail below.

Oral Fat Tolerance Test (OFTT)

Participants reported to the lab in the morning after a 12-hour overnight fast. A cannula was inserted into an antecubital or forearm vein to acquire blood samples.

After a blood sample was taken in the fasted state, a high fat meal comprising 150 ml cream, containing 75 g of fat, was ingested and further blood samples were collected at 30, 60, 90, 120, 240, 360 and 480 minutes after the meal. Participants were free to sit, read, relax, or watch TV during this time and were able to consume water ad libitum (see section 2.5.3).

Oral Glucose Tolerance Test (OGTT)

This trial was identical to the OFTT except participants ingested a drink containing 75 g of glucose, instead of the fat (see section 2.5.2).

OFTT and OGTT mixed meal

In this trial the participant consumed a mixture of 75g fat and 75g glucose (see section 2.5.4).

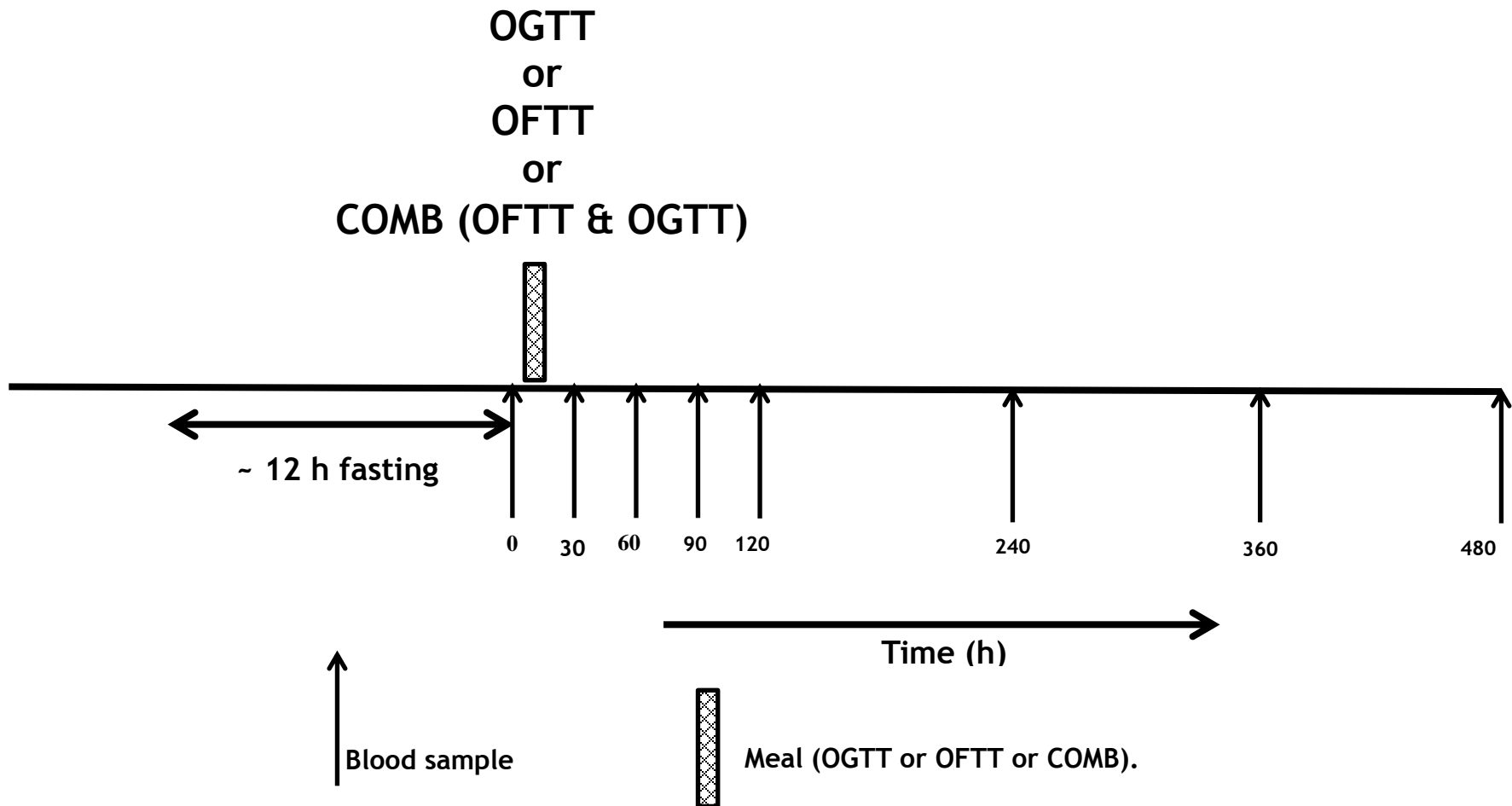


Figure 5.1. Study design. Participants reported to the lab after 12 h fasting. A base line blood sample was taken, then a meal containing either 75 g of fat or 75 g of glucose or combination of both was provided and serial blood samples were taken.

5.2.3 Plasma assays

Serum, TG and NEFA and plasma glucose concentrations were analysed at all time points as previously mentioned in **Appendix C**. Total, HDL cholesterol and small dense LDL concentrations were measured in the fasted state as mentioned in **Appendix C**. LDL cholesterol was calculated in the fasted state using the Friedewald equation (Friedewald et al., 1972). Insulin was measured in EDTA plasma, as described in **section 2.6.4**.

5.2.4 Lipoprotein separation

Plasma and serum samples (2 ml) at 0, 1, 2, 4, 6 and 8 h were centrifuged using the techniques to isolate chylomicrons and lipoprotein subfractions as described in **section 2.6.2**.

Chylomicron, VLDL₁ and VLDL₂ IDL and LDL fractions were assayed to determine concentrations and composition for TG, FC and PL using commercially available enzymatic and turbidimetric kits as described in **Appendix C**. Chylomicron fractions were assayed for TG concentration. Total protein was measured in all lipoprotein subfractions using a modified Lowry assay as described in **section 2.7**.

5.2.5 Calculations and statistical analysis

The rise in postprandial concentration was calculated by taking the incremental area under the curve (IAUC) and dividing by total time. Percentages of TG and CE were relative to lipoprotein total mass. TG to CE ratio was calculated as TG/CE.

Statistical analysis was carried out, Statistica (version 12.0) and Microsoft Excel (version 14.0.6112.500). When displayed graphically SEMs were used as error bars. Repeated measures ANOVAs were used to calculate differences in the means for time average concentrations, rise in time average concentrations, time to peak, the peak concentration and the maximal rise in concentration of plasma glucose, insulin, NEFA, TG and CM TG concentration and lipoprotein composition. Statistical significance was

set at $p < 0.05$ at which point a Fisher *post hoc* test was carried out to establish which groups the statistical significance lay. Cohen's d effect size was calculated to measure the magnitude of the co-ingestion of fat with carbohydrate (Cohen, 2013) (see section 2.9.5).

5.3 Results

5.3.1 Plasma measurements

Table 2.5 and Figure 5.2 show IAUC (\pm SEM) for plasma glucose over 480 minutes. The ingestion of carbohydrate alone and/or carbohydrate with fat increased the IAUC plasma glucose level when compare with ingesting fat only, in particular in the first 120 minutes. Glucose AUC and IAUC were significantly lower in OFTT compared with OGTT and COMB (all $p < 0.05$).

During the first 120 minutes observation, the consumption of fat with carbohydrate lowered both IAUC and AUC of the glycaemic response. Incremental glucose concentration in the first 2 hours was significantly higher in OGTT than in COMB by 49% (mean (95%CI) difference: $+0.55$ ($+0.04$ to $+1.06$) mmol.l^{-1} , $p=0.01$) and a moderate Cohen's effect size of 0.76 and in the AUC OGTT glucose concentrations were higher in OGTT then COMB by 12% with a mean (95%CI) difference: $+0.17$ ($+0.03$ to $+0.30$) mmol.l^{-1} , $p=0.03$). We observed a lower glycaemic response in COMB trial when compared with OGTT at 30 and 60 minutes. Glucose concentrations were lower by 52% ($p < 0.01$) and a moderate Cohen effect size of 0.74 at 30 minutes and by 65% ($p < 0.01$) and a large Cohen's effect size of 2.77.

Insulin response was not influenced by the addition of fat with carbohydrate compared to carbohydrate by itself (Table 2.5). Ingesting fat alone had a significantly lower effect on insulin secretion compared with the ingestion COMB test meal or OGTT test meal alone (all $p < 0.05$) (Figure 5.3).

The co-ingestion of fat with carbohydrate seems to have a suppressing effect on NEFA release. The IAUC is shown in Table 5.2 and Figure 5.4, at the first 30 minutes there greater reduction in NEFA concentration in the OGTT trial comparing with OFTT trial by 72% ($p < 0.01$) and in comparison with the COMB trial by 58% ($p < 0.03$). After an hour of ingestion, the reduction of NEFA continued to be lower in OGTT by 87% and 65% compared with both OFTT and COMB (all $p < 0.01$). At 90 minutes there was significant different between the three trials, as NEFA concentration continued to fall in OGTT

by 92% and 64% from both OFTT and COMB respectively (all $p < 0.01$) and the OFTT was lower by 85% compared with COMB ($p < 0.01$). After 2 hour of food ingestion NEFA concentration was lower in OGTT by 77% than OFTT and by 80% versus COMB (mean (95%CI) difference: $(-0.32 (-0.48 \text{ to } -0.17))$ giving a Cohen's d effect size of 1.27 and $(-0.23 (-0.40 \text{ to } -0.17))$ giving a Cohen's d effect size of 0.86 (all $p < 0.01$).

Plasma TG concentrations for incremental postprandial states for the group are shown in **Figure 5.5** and **Tables 5.2**. The co-ingestion of fat with carbohydrate did not have any significant effect on plasma TG concentrations. As expected OGTT trial was significantly different from both OFTT trial and COMB trial. The OGTT trial was different from OFTT trial by mean of (95%CI) difference: $-0.38 (-0.54 \text{ to } -0.22)$, and a large Cohen's d effect size of 1.47; and from COMB trial $(-0.39 (-0.54 \text{ to } -0.24))$ and a large Cohen's d effect size of 1.61 (all $p < 0.005$).

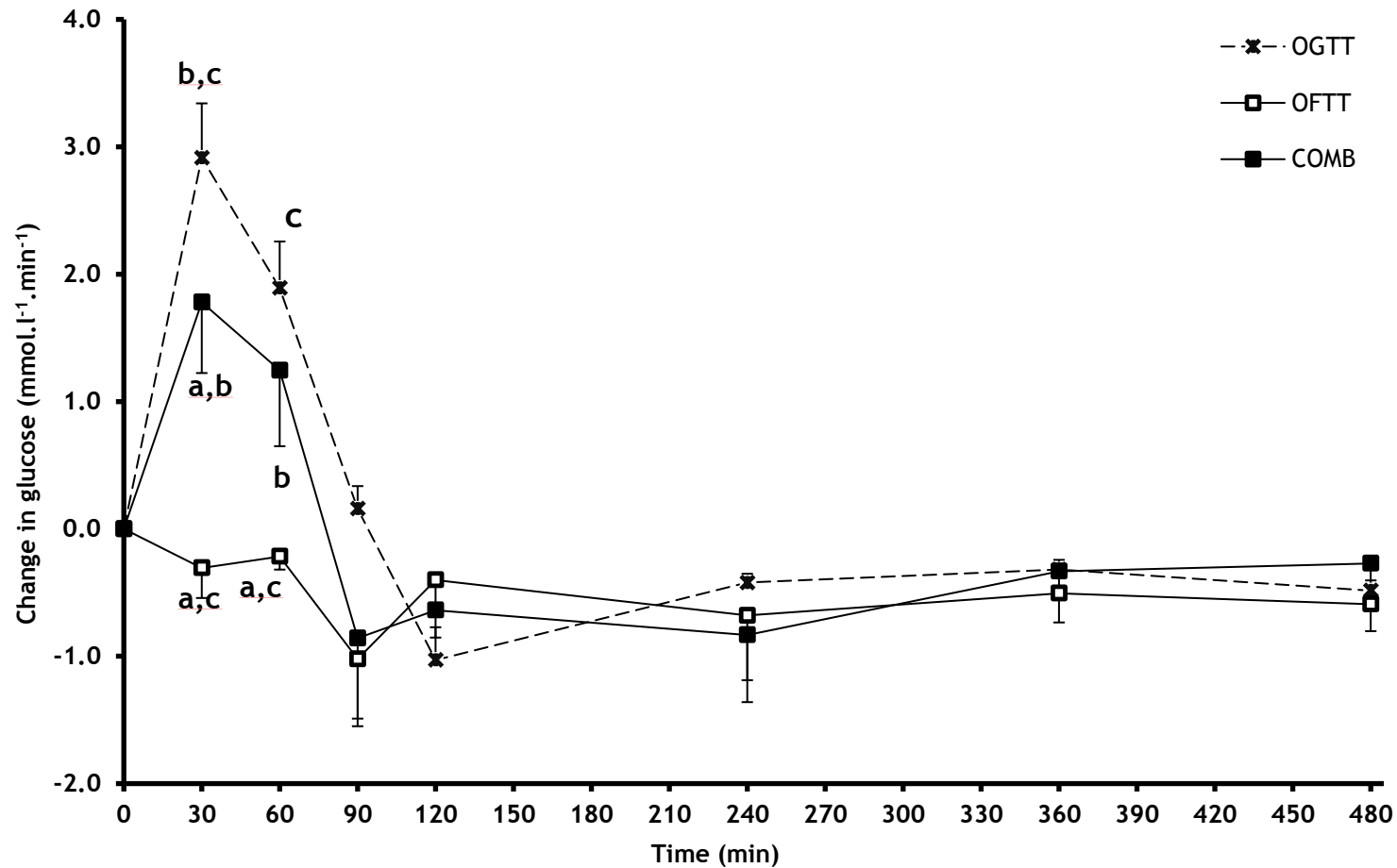


Figure 5.2. Change in glucose response over the 480 minute observation period in the OGTT, OFTT and COMB trials. Values are mean \pm SEM, N = 10; data were analysed using two ways ANOVA. Least significant differences post-hoc analysis was used to identify where significant main effect lay. ^aSignificantly different from OGTT trial, ^bsignificantly different from OFTT trial, ^csignificantly different from COMB trial, all ($p < 0.001$).

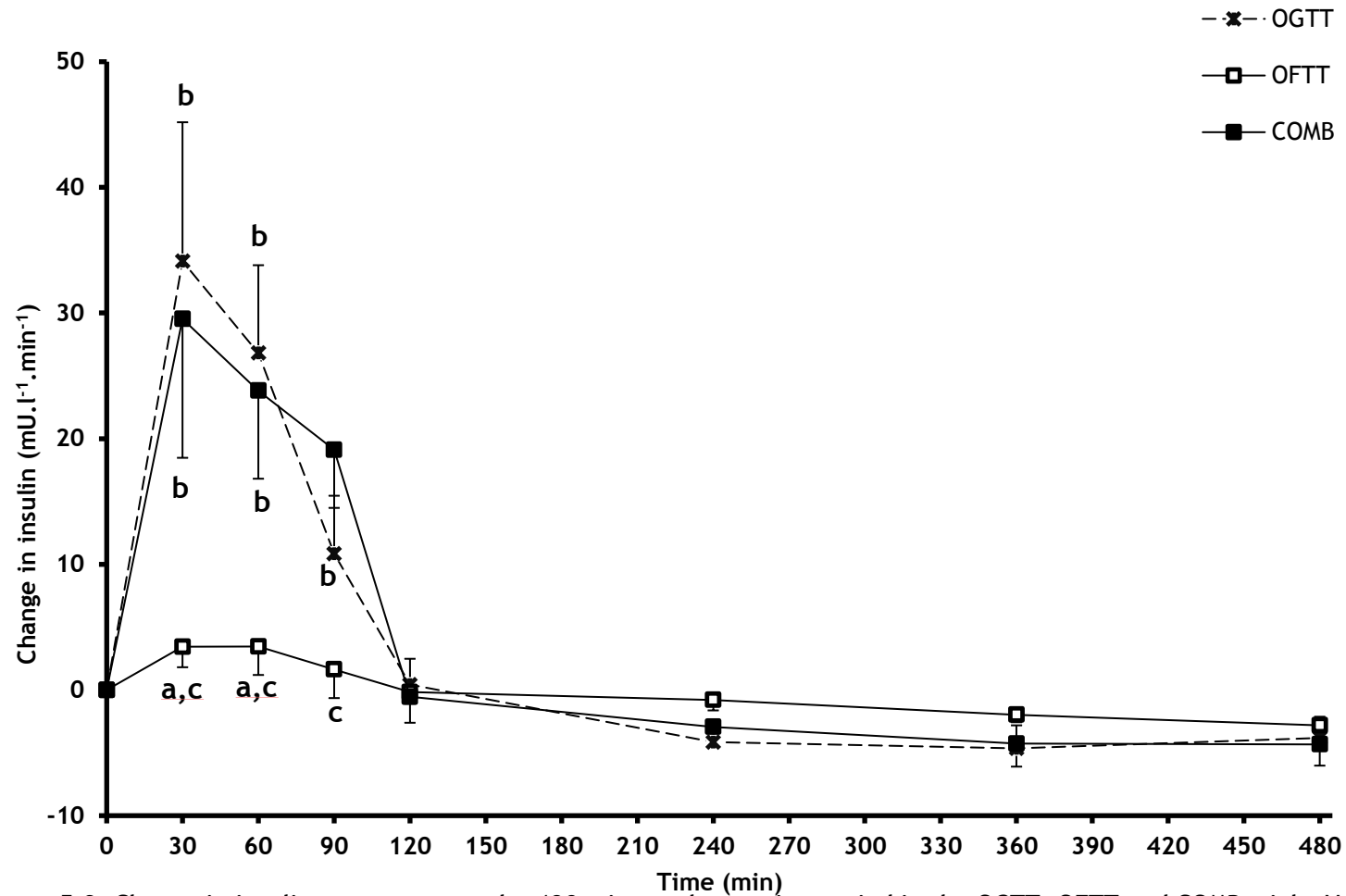


Figure 5.3. Change in insulin response over the 480 minute observation period in the OGTT, OFTT and COMB trials. Values are mean \pm SEM, N = 10; data were analysed using two ways ANOVA. Least significant differences post-hoc analysis was used to identify where significant main effect lay ^aSignificantly different from glucose trial, ^bsignificantly different from fat trial, ^csignificantly different from combination trial, all ($p < 0.001$).

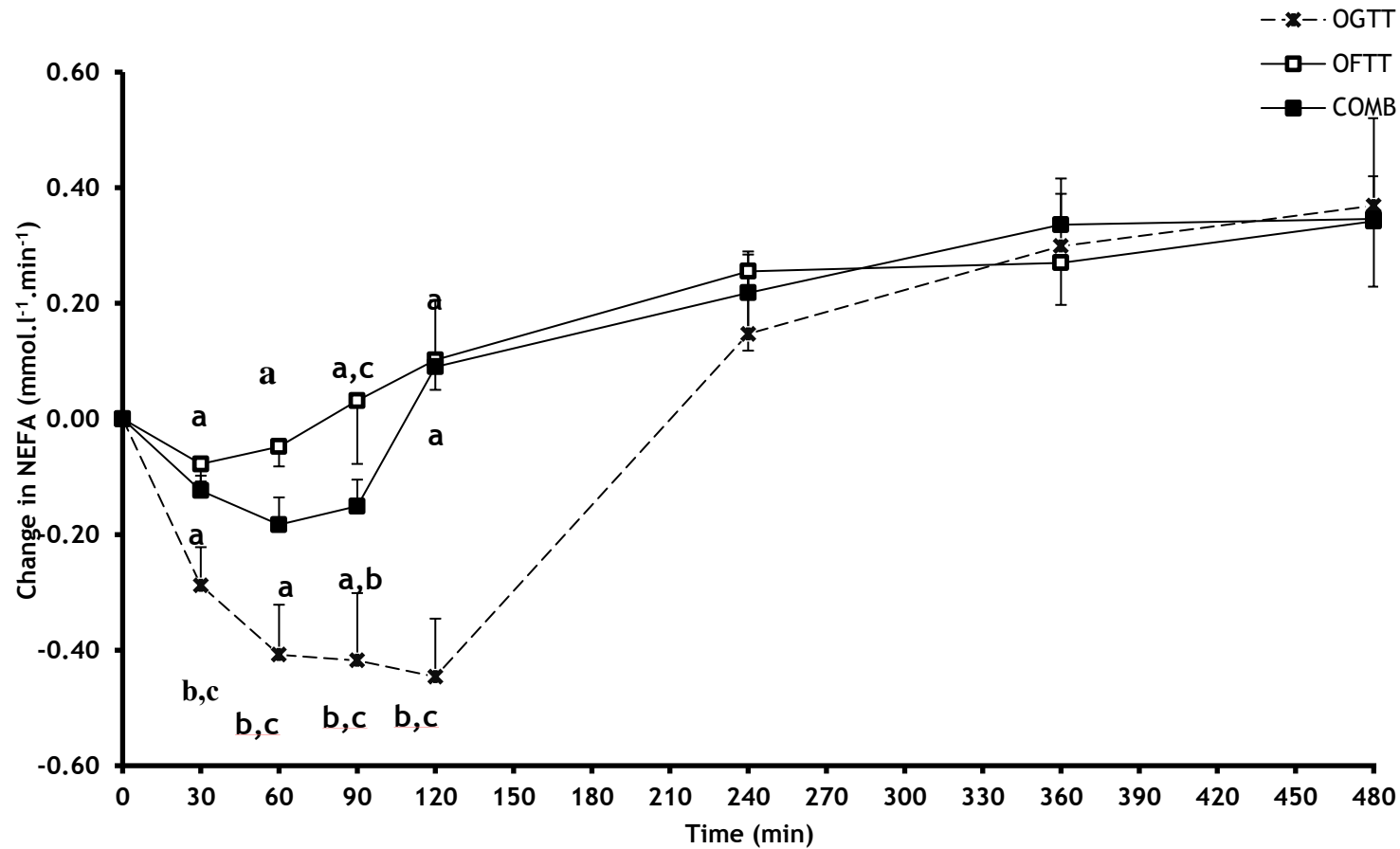


Figure 5.4. Change in NEFA response over the 480 minute observation period in the OGTT, OFTT and COMB trials. Values are mean \pm SEM, N = 10; data were analysed using two ways ANOVA. Least significant differences post-hoc analysis was used to identify where significant main effect lay ^aSignificantly different from glucose trial, ^bsignificantly different from fat trial, ^csignificantly different from combination trial, all ($p < 0.001$).

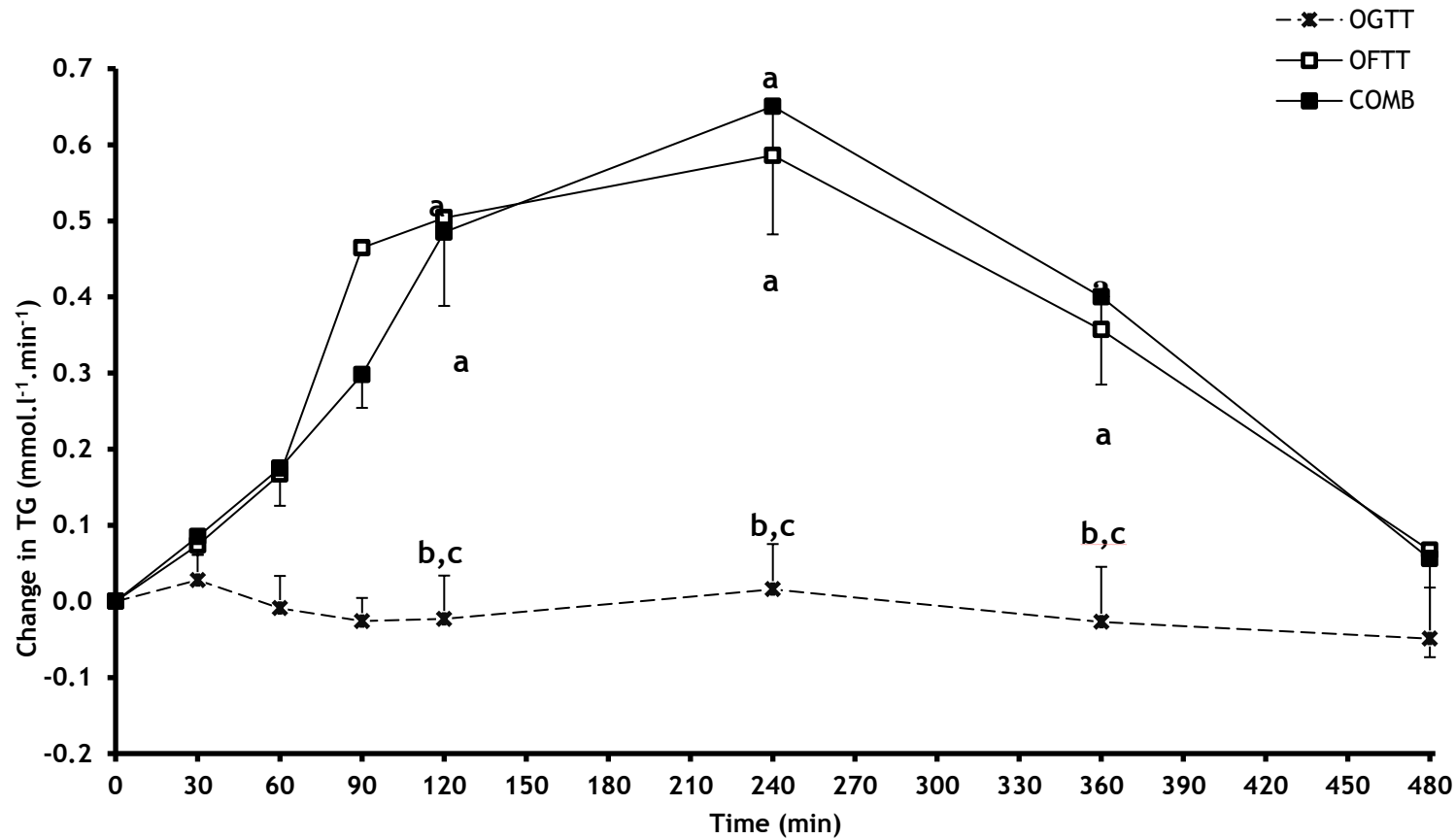


Figure 5.5. Change in TG response over the 480 minute observation period in the OGTT, OFTT and COMB trials. Values are mean \pm SEM, N = 10; data were analysed using two ways ANOVA. Least significant differences post-hoc analyses was used to identify where significant main effect lay ^aSignificantly different from glucose trial, ^bsignificantly different from fat trial, ^csignificantly different from combination trial, all ($p < 0.001$).

Table 5.2; Change in plasma values over the 480-minute postprandial observation period in the OGTT, OFTT and COMB trials.

	Mean Value			Mean difference			Effect Size		
	OGTT	OFTT	COMB	OGTT vs OFTT	OGTT vs COMB	OFTT vs COMB	OGTT vs OFTT	OGTT vs COMB	OFTT vs COMB
TG (mmol.l ⁻¹)	-0.01 ± 0.05	0.37 ± 0.08	0.38 ± 0.08	-0.38 (-0.54 to -0.22)	-0.39 (-0.54 to -0.24)	+0.01 (-0.13 to +0.16)	1.47**	1.61**	0.06
NEFA (mmol.l ⁻¹)	0.02 ± 0.12	0.18 ± 0.06	0.17 ± 0.05	-0.17 (-0.36 to +0.03)	-0.15 (-0.37 to +0.07)	-0.02 (-0.10 to +0.07)	0.52	0.41	0.12
Glucose (mmol.l ⁻¹)	-0.10 ± 0.08	-0.35 ± 0.15	-0.19 ± 0.12	+0.26 (+0.02 to +0.49)	+0.09 (-0.18 to +0.36)	+0.17 (-0.22 to +0.56)	0.68**	0.21*	0.26
First h glucose (mmol.l ⁻¹)	1.11 ± 0.18	-0.29 ± 0.15	0.56 ± 0.21	+1.38 (+1.03 to +1.7)	+0.54 (+0.01 to +1.05)	+0.58 (+0.34 to +1.35)	2.47**	0.64*	1.04**
Insulin (mU.l. ⁻¹)	1.84 ± 2.47	-0.48 ± 0.67	2.28 ± 4.22	+2.32 (-3.36 to +8.00)	-0.45 (-6.31 to +5.41)	+2.76 (-5.70 to +11.2)	0.25	0.05	0.20
First h insulin (mU.l ⁻¹)	17.84 ± 5.76	2.34 ± 1.16	18.05 ± 8.69	+15.52 (+3.60 to +27.42)	-0.19 (-11.06 to +10.62)	+15.71 (-1.21 to +32.62)	0.81**	0.01	0.58**

Values are the mean ± SEM, n=10; data were analysed using repeated measure ANOVA.* significant from the other group p<0.05, **p<0.005

5.3.2 Lipoprotein composition

Triglyceride rich lipoprotein

Table 5.3 shows the AUC (\pm SEM) values of TG concentration in chylomicron particles in the three trials. The pattern of TG IAUC in chylomicron was similar to TG IAUC in plasma (**Figure 5.7**). There was a significant difference between the three trials and the time points in each trial and the pattern of the change (all $p < 0.001$). After food ingestion, within an hour the TG concentration started to rise similarly in both OFTT and COMB trials. The mean IAUC values of chylomicron-TG were significantly lower in OGTT (mean (95%CI) difference: -0.19 (-0.24 to -0.13, $p < 0.005$)) compared with OFTT, giving a large Chen's d effect size of 1.84, and in OGTT compared with COMB (mean (95%CI) difference: -0.13 (-0.05 to + 0.04, $p < 0.005$)). However, there was no significant difference between IAUC chylomicron-TG concentration in OFTT and COMB trials.

The ingestion of carbohydrate alone and the co-ingestion of carbohydrate seem to increase the TG percentage in VLDL₁ particles (**Figure 5.7 panel A and Table 5.3**). Whereas, if fat was ingested alone TG concentrations were significantly lower than OGTT and COMB trials respectively by 21% (+10.14 (0.04 to +20.33) giving a moderate Cohen's effect size of 0.62 and by 21% (+10.23 (-0.63 to +21.09) giving a moderate Cohen's effect size 0.58 (all $p = 0.03$).

The percentage of phospholipid was higher in OFTT trial when compared with OGTT and COMB trials. Phospholipid in OFTT trial was significantly higher by 19% from OGTT trial, giving a moderate Cohen's d effect size of 0.55 ($p = 0.056$) and 18% comparing OFTT with COMB trial, giving a moderate Cohen's d effect size of 0.56 ($p = 0.06$).

VLDL₁ particles incremental concentration seemed to increase by approximately double when fat alone was ingested (**Figure 5.8 panel A**). The co-ingestion of fat with carbohydrate and/ or ingestion of carbohydrate alone, reduced VLDL₁ concentrations by 45%, giving a large Cohen's d effect size of 0.82 and 59%, giving a small Cohen's d effect size of 0.42 respectively when compared with ingesting fat alone (**Table 5.3**). However, at 8 hours of meal ingestion, the concentration of VLDL₁

in COMB trial decreased dramatically when compared with OGTT mass by 158% ($p < 0.05$).

There was no any significant difference in the percentage of FC, CE, protein and CE/TG ratio between the three meal types (see **Table 5.3**).

The co-ingestion of fat with carbohydrate had a small influence on VLDL₂ composition. There was no significant different in the percentage TG, CE, FC, PL and protein between the three trials (see **Table 5.4**).

There was a trend of reduction in VLDL₂ concentration when fat alone ingested. In OFTT trial VLDL₂ concentration was higher than OGTT trial by 67%, giving a moderate Cohen's d effect size 0.43 and COMB trial by 69%, respectively.

The CE/TG ratio was double in OFTT significantly comparing with OGTT and COMB and giving a small Cohen's d effect size of 0.55 and 0.61 respectively (all $p = 0.05$) (see **Table 5.4** and **Figure 5.8**, panel B and **Figure 5.10**).

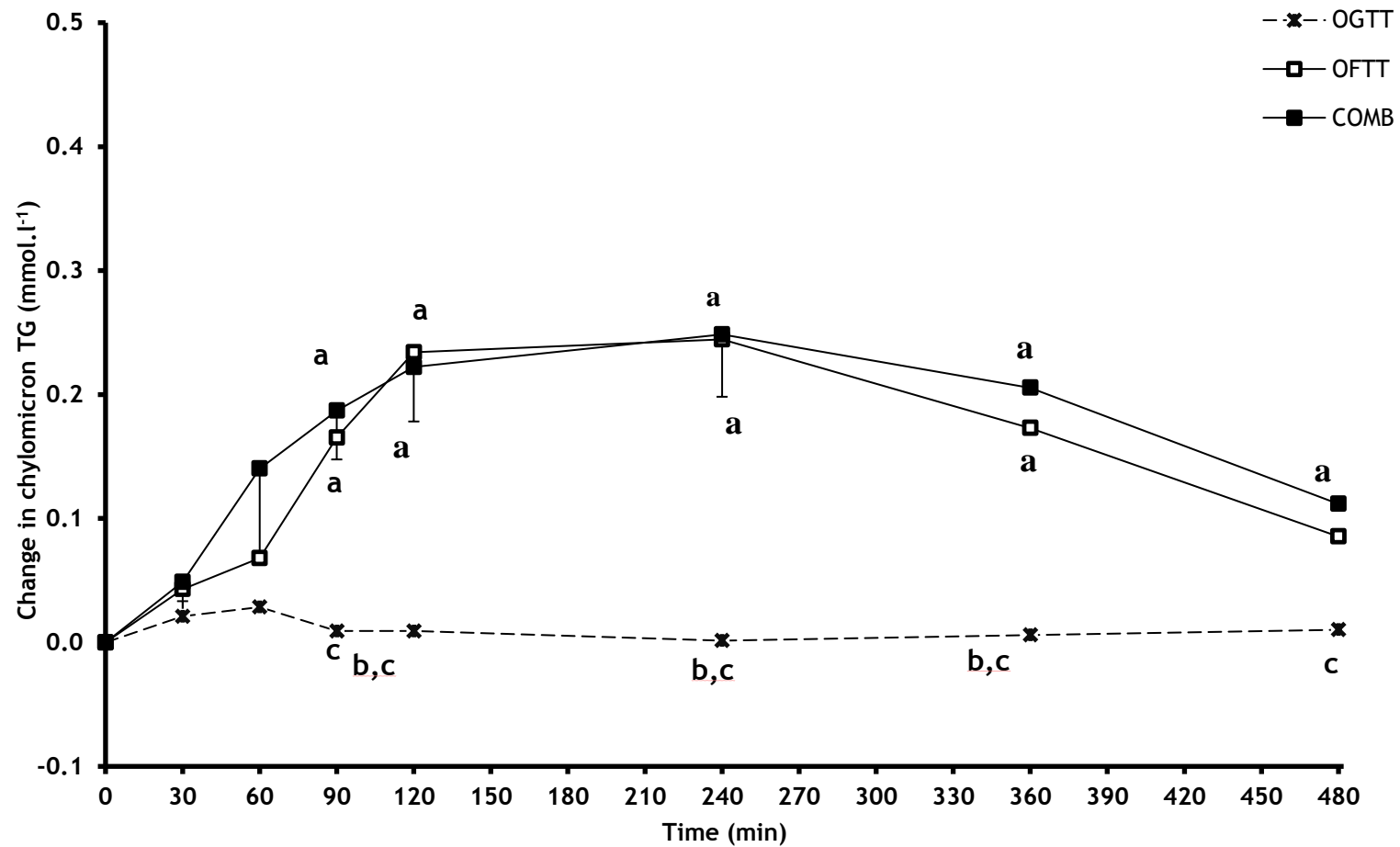


Figure 5.6. Change in chylomicron-TG response over the 480 minute observation period in the OGTT, OFTT and COMB trials. Values are mean \pm SEM, N = 10; data were analysed using two ways ANOVA. Least significant differences post-hoc analysis was used to identify where significant main effect lay ^aSignificantly different from glucose trial, ^bsignificantly different from fat trial, ^csignificantly different from combination trial, all ($p < 0.001$).

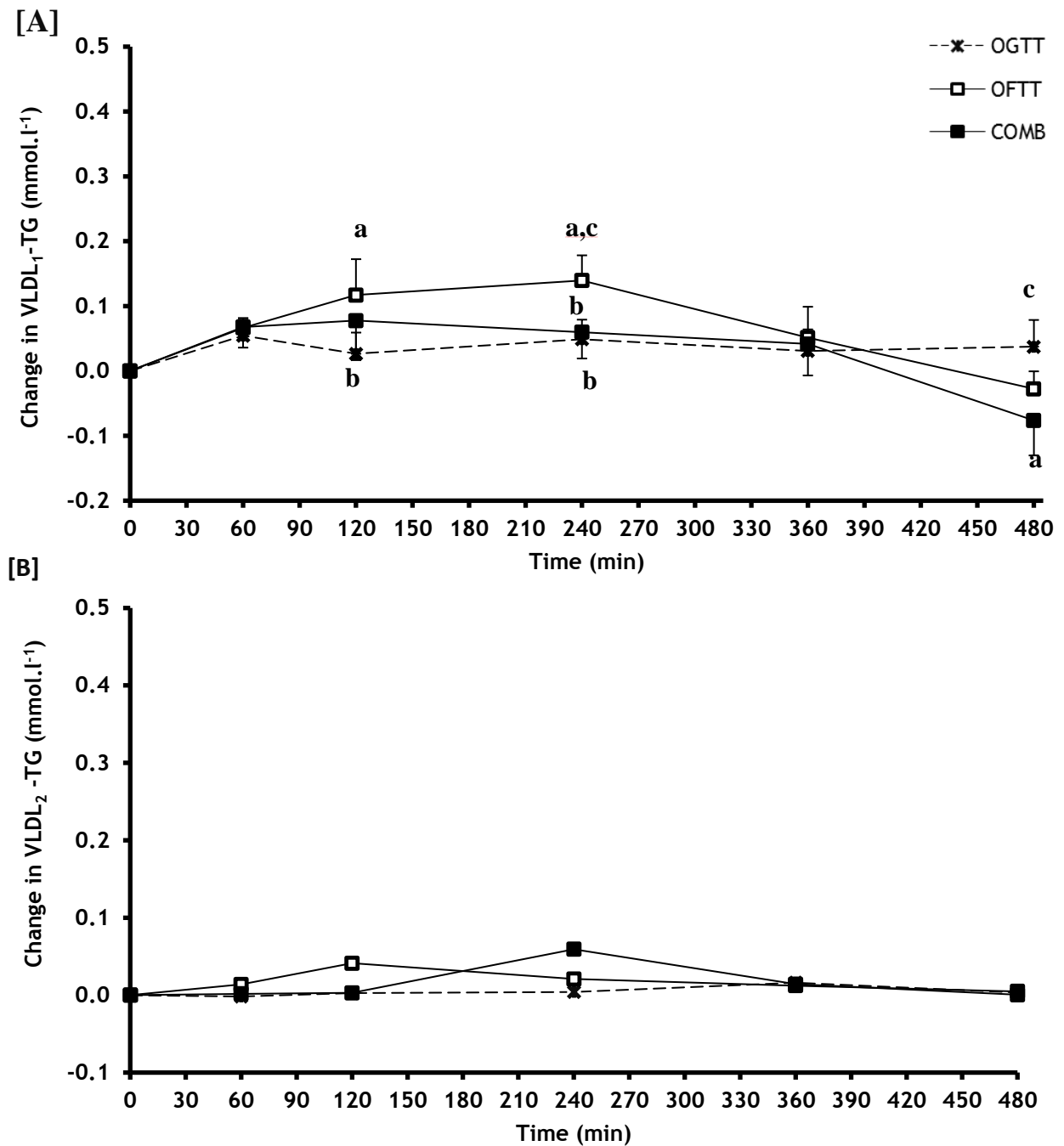


Figure 5.7. Change in lipoprotein-TG response over the 480 minute observation period, panel [A] shows the change in VLDL₁-TG and panel [B] the change in VLDL₂-TG in the OGTT, OFTT and COMB trials. Values are mean \pm SEM, n = 10; data were analysed using two ways ANOVA. Least significant differences post-hoc analysis was used to identify where significant main effect lay ^aSignificantly different from glucose trial, ^bsignificantly different from fat trial, ^csignificantly different from combination trial, all (p<0.05).

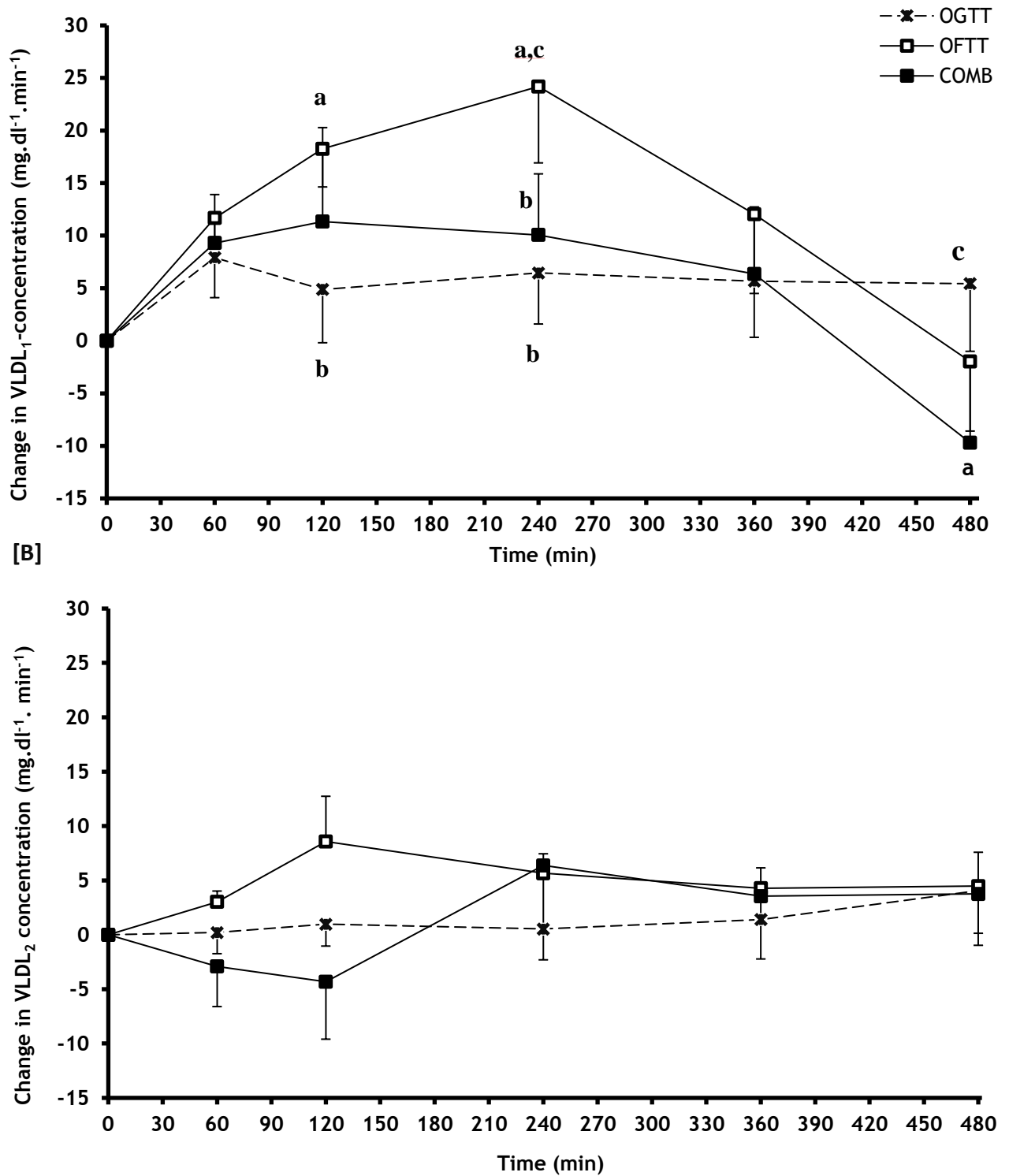


Figure 5.8. Change in lipoprotein concentration response over the 480 minute observation period, panel [A] shows the change in VLDL₁ concentration and panels [B] the change in VLDL₂ concentration in the OGTT, OFTT and COMB trials. Values are mean \pm SEM, n= 10; data were analysed using two ways ANOVA. Least significant differences post-hoc analysis was used to identify where significant main effect lay ^aSignificantly different from glucose trial, ^bsignificantly different from fat trial, ^csignificantly different from combination trial, all (p<0.05).

Table 5.3; Time-averaged areas under the curve for chylomicron concentration and VLDL₁ composition and concentration values over the 480-minute postprandial observation period in the OGTT, OFTT and COMB trials.

	Mean Value			Mean difference			Effect size		
	OGTT	OFTT	COMB	OGTT vs OFTT	OGTT vs COMB	OFTT vs COMB	OGT T vs OFTT	OGTT vs COMB	OFTT vs COMB
Chylomicron TG (mmol.l ⁻¹)	0.03 ± 0.01	0.22 ± 0.03	0.22 ± 0.03	-0.19 (-0.24 to -0.13)	-0.19 (-0.05 to +0.04)	0.00 (+2.50 to +9.22)	2.15*	2.30**	0.04
IAUC total lipoprotein concentration (mg.dl ⁻¹)	5.94 ± 4.11	13.65 ± 4.18	6.18 ± 4.82	-7.71 (-8.29 to -1.91)	-0.24 (-1.38 to +11.20)	-7.47 (-8.58 to +3.58)	0.82	0.01	0.42
Triglyceride (%)	58.56 ± 1.27	48.42 ± 4.97	58.65 ± 2.10	+10.14 (+0.04 to +20.33)	+0.24 (-2.62 to +3.11)	+10.23 (-0.63 to +21.09)	0.62*	0.05	0.58*
Free cholesterol (%)	4.67 ± 0.67	5.41 ± 0.85	5.43 ± 0.28	-0.74 (-2.56 to +1.07)	-0.76 (-1.77 to +0.25)	+0.02 (-1.40 to +1.44)	0.25*	0.47	0.01
Cholesteryl ester (%)	11.11 ± 1.71	15.21 ± 4.11	10.96 ± 1.77	-4.11 (-13.03 to +4.81)	+0.15 (-1.67 to +1.96)	-4.26 (-13.94 to +5.42)	0.29	0.05	0.27
Phospholipid (%)	15.11 ± 0.80	18.34 ± 1.78	15.43 ± 0.61	-3.23 (-6.84 to +0.38)	-0.23 (-1.69 to +1.04)	-2.91 (-6.16 to +0.34)	0.55	0.15	0.56*
Protein (%)	10.55 ± 0.84	12.62 ± 2.72	9.53 ± 1.11	-2.06 (-7.22 to +3.10)	+1.03 (-1.96 to +4.01)	-3.09 (-7.15 to +0.98)	0.25	0.21	0.47
CE/TG (mol:mol)	0.26 ± 0.04	0.67 ± 0.34	0.28 ± 0.06	-0.41 (-0.47 to +0.27)	-0.04 (-0.02 to +0.04)	+0.39 (+0.32 to +1.10)	0.37	0.17	0.34

Values for lipoprotein concentration is mean IAUC ± SEM, rest of the values are the mean ± SEM, n=10; data were analysed using repeated measure ANOVA.* significant from the other group p<0.05, **p<0.005.

Table 5.4; Time-averaged areas under the curve for VLDL₂ composition and concentration values over the 480-minute postprandial observation period in the OGTT, OFTT and COMB trials.

	Mean Value			Mean different			Effect Size		
	OGTT	OFTT	COMB	OGTT vs. OFTT	OGTT vs. COMB	OFTT vs. COMB	OGTT vs. OFTT	OGTT vs. COMB	OFTT vs. COMB
IAUC total lipoprotein concentration (mg.dl ⁻¹)	1.20 ± 2.55	5.03 ± 1.55	1.40 ± 3.46	-3.83 (-4.38 to +1.64)	-0.20 (-0.98 to +7.55)	-3.63 (-4.32 to +3.26)	0.43	0.02	0.33
Triglyceride (%)	39.44 ± 2.80	33.24 ± 2.98	34.70 ± 3.54	+6.20 (-2.76 to +15.16)	+4.74 (+0.45 to +9.04)	+1.46 (-7.63 to +10.55)	0.43	0.68	0.10
Free cholesterol (%)	7.72 ± 0.87	8.40 ± 0.49	8.75 ± 0.62	-0.68 (-2.31 to +0.95)	-1.03 (-2.16 to +0.09)	+0.36 (-0.88 to +1.59)	0.26	0.57	0.18
Cholesteryl ester (%)	18.01 ± 1.60	17.31 ± 1.89	19.35 ± 2.14	+0.69 (-2.20 to +3.59)	-1.34 (-5.08 to +2.39)	+2.04 (-2.61 to +6.68)	0.15	0.22	0.27
Phospholipid (%)	17.79 ± 1.63	20.45 ± 1.40	19.16 ± 1.07	-2.65 (-6.45 to +1.15)	-1.37 (-3.24 to +0.50)	-1.29 (-3.78 to +1.21)	0.43	0.45	0.32
Protein (%)	17.03 ± 2.28	20.60 ± 5.93	18.04 ± 2.91	-3.57 (-15.49 to +8.35)	-1.00 (-4.32 to +2.32)	-2.57 (-13.02 to +7.89)	0.19	0.19	0.15
CE/TG ratio (mol:mol)	0.48 ± 0.07	1.11 ± 0.37	0.67 ± 0.11	-0.63 (-0.70 to +0.08)	-0.19 (-0.21 to -0.01)	+0.44 (-0.37 to +1.20)	0.55*	0.61*	0.36

Values for lipoprotein concentration is mean IAUC ± SEM, rest of the values are the mean ± SEM, n=10; data were analysed using repeated measure ANOVA.* significant from the other group p<0.05, **p<0.005.

Cholesterol-rich lipoproteins

Table 5.5 shows the AUC (\pm SEM) values of shows mean AUC (\pm SEM) of IDL percentage composition. The Carbohydrate ingestion led to enrich the particle with TG when compared to the two trials. However, the ratio of CE/TG did not differ significantly. Fat ingestion; lead the particle to have more phospholipids. There was no significant different in, CE, FC, protein and total lipoprotein mass (**Figure 5.9, panel A and Figure 5.10**).

Table 5.6 shows mean AUC (\pm SEM) of LDL percentage composition. Ingesting carbohydrate alone increased TG concentration and reduced phospholipid concentration in LDL particles when compared with ingesting fat alone and/or co-ingesting fat with carbohydrate. As shown in **Table 5.6** TG concentration in OGTT trial was higher by 52% comparing with ingesting fat alone and/or the co-ingestion of both by 49% (all $p=0.05$).

On the other hand, phospholipid was lower in OGTT trial by 12% and 9% comparing with both OFTT and COMB trials respectively, giving a moderate Cohen's d effect size of 0.57 and 0.54 ($p=0.3$ and $p=0.05$) respectively. There was a trend of lower CE/TG ratio by 29% when carbohydrate alone was ingested comparing to ingesting fat alone, giving a moderate Cohen's d effect size of 0.71. There was no significant different in, CE, FC, protein and total lipoprotein mass (**Figure 5.9, panel B and Figure 5.10**). The summary of lipoproteins result is shown in **Figure 5.10**, when carbohydrate was ingested alone or co-ingested with fat, both follow similar pattern on lipoprotein composition during 480 minutes of observation. Number of particles seem lower, however, they become more TG enrich.

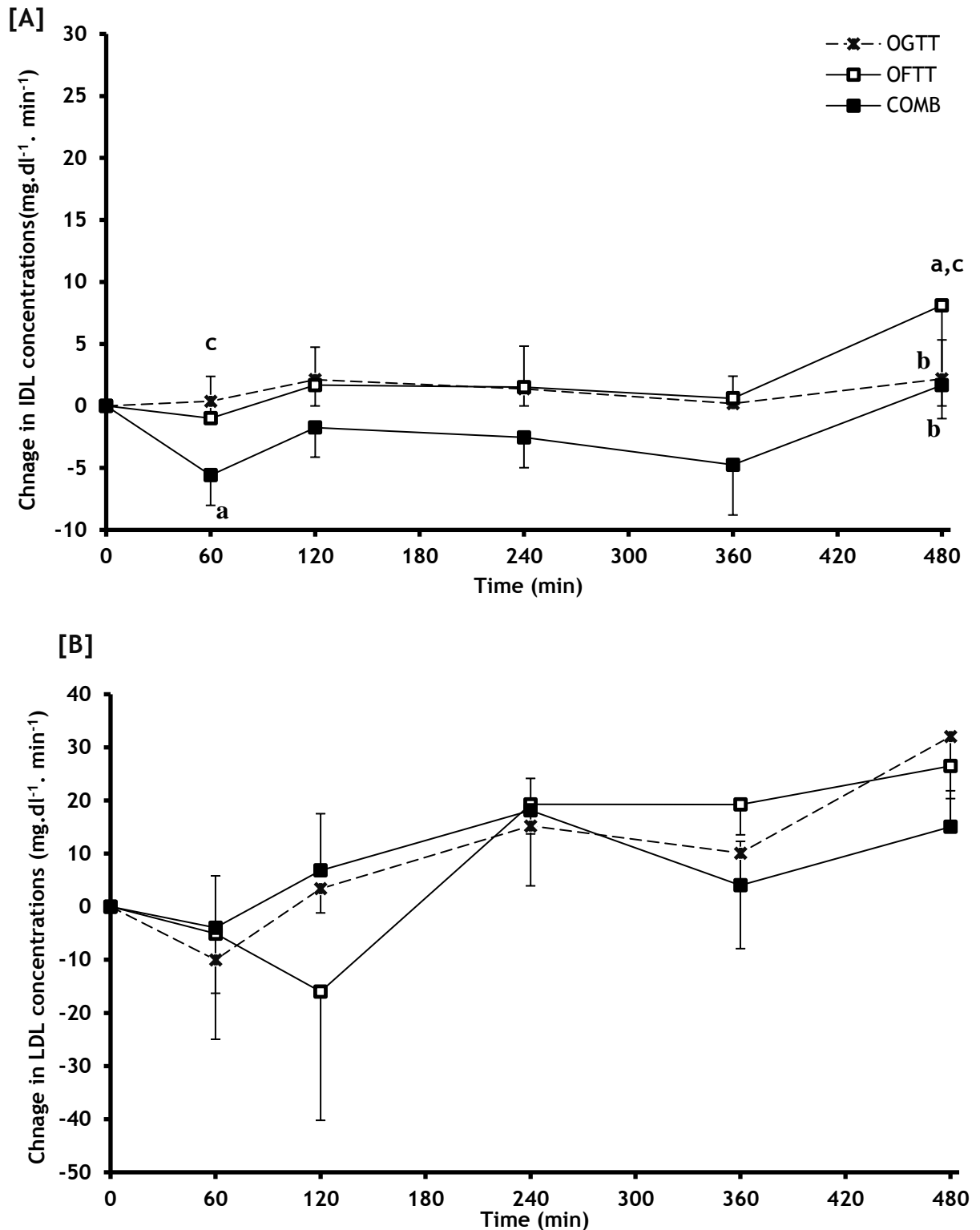


Figure 5.9. Change in lipoprotein concentration response over the 480 minute observation period, panel [A] shows the change in IDL concentration and panel [B] shows the change in LDL concentration in the OGTT, OFTT and COMB trials. Values are mean \pm SEM, $n = 10$; data were analysed using two ways ANOVA. Least significant differences post-hoc analysis was used to identify where significant main effect lay ^aSignificantly different from glucose trial, ^bsignificantly different from fat trial, ^csignificantly different from combination trial, all ($p < 0.05$).

Table 5.5; Time-averaged areas under the curve for IDL composition and concentration values over the 480-minute postprandial observation period in the OGTT, OFTT and COMB trials.

	Mean value			Mean different			Effect size		
	OGTT	OFTT	COMB	OGTT vs. OFTT	OGTT vs. COMB	OFTT vs. COMB	OGTT vs. OFTT	OGTT vs. COMB	OFTT vs. COMB
Total lipoprotein concentration (mg.dl ⁻¹)	1.11 ± 2.30	1.74 ± 2.72	-2.64 ± 1.90	-0.63 (-5.64 to +4.39)	+3.75 (-2.53 to +10.03)	-4.38 (-10.98 to +2.22)	0.08	0.37	0.41
Triglyceride (%)	14.64 ± 2.66	10.42 ± 0.71	9.78 ± 0.97	+4.22 (-0.20 to +8.63)	+4.86 (-0.09 to +9.81)	-0.64 (-2.80 to +1.52)	0.59*	0.61*	0.18
Free cholesterol (%)	13.31 ± 1.73	12.65 ± 0.67	12.31 ± 0.78	+0.65 (-2.51 to +3.18)	+1.00 (-2.53 to +4.52)	0.34 (-1.95 to +1.27)	0.13	0.18	0.13
Cholesteryl ester (%)	32.13 ± 3.07	35.83 ± 2.46	36.92 ± 1.57	-3.70 (-9.45 to +2.05)	-4.79 (-11.86 to +2.27)	+1.09 (-5.22 to +7.40)	0.40	0.42	0.11
Phospholipid (%)	21.81 ± 1.67	25.09 ± 0.60	22.87 ± 0.69	-3.27 (-7.22 to +0.67)	-1.05 (-4.46 to +2.35)	-2.22 (-3.95 to -0.49)	0.51*	0.19	0.80*
Protein (%)	18.15 ± 2.13	16.06 ± 1.86	18.12 ± 2.34	+2.09 (-1.43 to +5.61)	+0.04 (-5.53 to +5.60)	+2.05 (-3.48 to +7.58)	0.37	0.00	0.23
CE/TG ratio (mol:mol)	4.21 ± 0.87	5.05 ± 0.67	5.77 ± 0.78	-0.84 (-0.98 to +0.56)	-1.56 (-1.78 to +0.68)	-0.72 (-0.90 to +1.11)	0.37	0.43	0.24

Values for lipoprotein concentration is mean IAUC ± SEM, rest of the values are the mean ± SEM, n=10; data were analysed using repeated measure ANOVA.* significant from the other group p<0.05, **p<0.005.

Table 5.6; Time-averaged areas under the curve for LDL composition and concentration values over the 480-minute postprandial observation period in the OGTT, OFTT and COMB trials.

	Mean value			Mean different			Effect size		
	OGTT	OFTT	COMB	OGTT vs. OFTT	OGTT vs. COMB	OFTT vs. COMB	OGTT vs. OFTT	OGTT vs. COMB	OFTT vs. COMB
Total lipoprotein concentration (mg.dl ⁻¹)	9.73 ± 9.22	9.31 ± 7.70	8.21 ± 4.44	+0.41 (-26.72 to +27.55)	+1.52 (-20.40 to +21.05)	-1.10 (-19.01 to +16.80)	0.02	0.01	0.04
Triglyceride (%)	8.29 ± 2.11	4.65 ± 0.32	4.83 ± 0.37	+3.73 (+0.10 to +7.36)	+3.46 (+0.41 to +7.32)	+0.27 (-0.40 to +0.95)	0.64*	0.55*	0.40
Free cholesteryl (%)	10.64 ± 2.28	10.72 ± 0.63	11.08 ± 0.55	-0.08 (-4.40 to +4.24)	-0.44 (-4.99 to +4.12)	+0.35 (-1.13 to +1.84)	0.01	0.006	0.15
Cholesterol ester (%)	35.74 ± 1.81	37.19 ± 1.22	37.62 ± 1.39	-1.45 (-5.48 to +2.59)	-1.88 (-6.20 to +2.44)	+0.44 (-2.93 to +3.80)	0.22	0.27	0.08
Phospholipid (%)	19.23 ± 0.93	21.59 ± 0.69	20.99 ± 0.36	-2.36 (-4.94 to +0.23)	-1.76 (-3.77 to +0.26)	-0.60 (-1.87 to +0.67)	0.57*	0.54*	0.29
Protein (%)	26.09 ± 1.43	25.94 ± 1.77	25.47 ± 1.48	+0.15 (-3.22 to +3.52)	+0.62 (-3.20 to 4.43)	-0.47 (-3.86 to +2.93)	0.03	0.10	0.08
CE/TG ratio (mol:mol)	9.12 ± 1.56	11.81 ± 11.02	11.31 ± 1.07	-2.69 (-2.92 to -0.33)	-2.19 (-2.49 to +0.85)	+0.50 (+0.31 to +2.45)	0.71	0.45	0.16

Values for lipoprotein concentration is mean IAUC ± SEM, rest of the values are the mean ± SEM, n=10; data were analysed using repeated measure ANOVA.* significant from the other group p<0.05, **p<0.005.

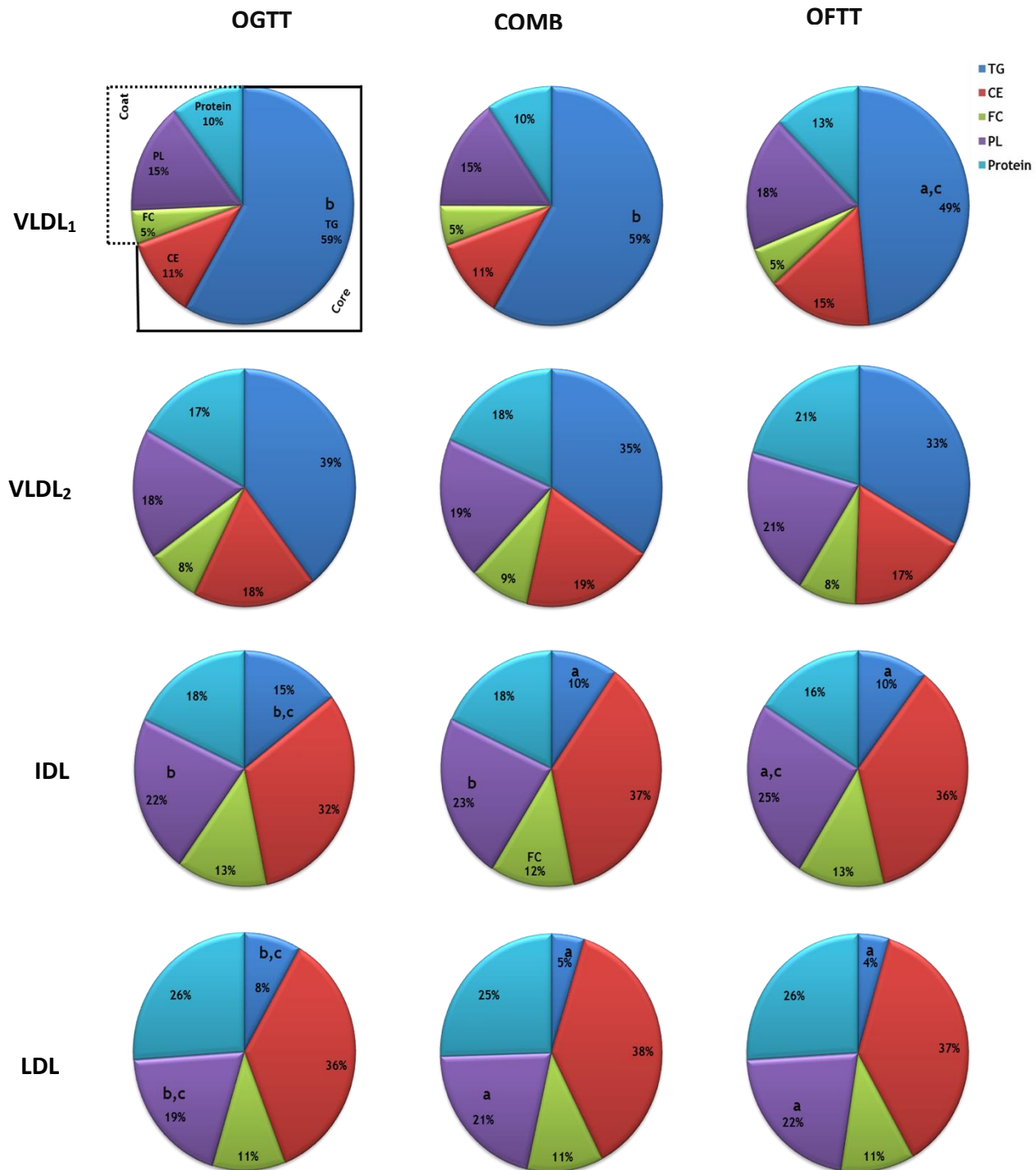


Figure 5.10. Differences between lipoprotein compositions in the three trials, lay ^aSignificantly different from glucose trial, ^bsignificantly different from fat trial, ^csignificantly different from combination trial, $p < 0.005$. Protein, phospholipid (PL) and free-cholesterol (FC) comprise the outer coat of the lipoprotein; triglyceride (TG) and cholesteryl-ester (CE) comprise the lipoprotein core.

5.4 Discussion

The goal of this study was to address the effect of ingesting fat and carbohydrate separately and in combination on postprandial responses. The results of the experiments appear are in line with existing literature (Wilson et al., 1985, Pedersen et al., 1999, Knuth et al., 2008, Collier and O'Dea, 1983).

Glycaemic Response

It is well established that glucose intake increases plasma insulin (Elrick et al., 1964, Stumvoll et al., 2000). The finding of the current study with glucose ingestion is in line with this finding. Hyperglycaemia is a major contributor to endothelial damage providing a link between CVD and T2D (Ceriello, 2004). In contrast OFTT meal led to a low glycaemic and insulinemic response, in line with previously observations (Riccardi et al., 2004, Havel, 1957a).

In the current study, adding equal amounts of fat and carbohydrate (75 grams each) reduced the glycaemic response compared to carbohydrate ingestion alone. However, this response will be influenced by the amounts of fat and / or carbohydrate consumed, for example, in studies in which 8-24 g fat was fed in mixed meals containing 38-104 g carbohydrate, the added fat had little effect on the glycaemic response (Wolever and Bolognesi, 1996a). It has been observed that an intake of 15 g of fat has no influence on postprandial lipaemia and lipoproteins in healthy adults (Dubois et al., 1998). The current data shows that the COMB meal significantly reduced the glucose response, compared to carbohydrate ingestion alone, particularly during the first hour after meal ingestion. This is as has observed previously (Collier et al., 1984, Collier and O'Dea, 1983, Collier et al., 1988). The most likely explanation for these results is that fat slows gastric emptying (Thomas, 1957, Gentilcore et al., 2006, Heddle et al., 1989, Phillips et al., 2015) Gastric emptying is an important determinant of rate of glucose appearance and blood glucose homeostasis in healthy and diabetic populations (Horowitz et al., 1993). This is mediated by gut hormones such as gastric inhibitory polypeptide and glucagon-like peptide-1 (GLP-1) (Rocca and Brubaker, 1995, Herrmann et al., 1995, Feltrin et al.,

2004). The reduction of the rate of glucose absorption after the consumption of the mixture of carbohydrate with fat will attenuate glycaemic and insulinaemic responses (Gannon et al., 1988, Pi-Sunyer, 2002) and reduce the postprandial rise in some hormones such as incretins, and insulin (Jenkins et al., 2002, Ludwig, 2002). The prolonged absorption of carbohydrate seen over time will maintain suppression of the free fatty acids and the counter regulatory responses, resulting in slower carbohydrate entry into the small intestine, while at the same time achieving lower blood glucose concentrations (Jenkins et al., 1990, Jenkins et al., 1988, Wolever et al., 1988, Ludwig et al., 1999). However, COMB meal did not lower the insulin AUC significantly and this has been observed previously (Collier et al., 1984, Collier and O'Dea, 1983, Collier et al., 1988). This is might be due to the type of dietary fat in particular saturated fatty acid which also affects insulin sensitivity, independently of its effects on body weight (Jenkins et al., 1978, Mayer et al., 1993), while monounsaturated and polyunsaturated fatty acids improve it through modifications in the composition of cell membranes (Chen et al., 1988, Swinburn et al., 1991, Lovejoy et al., 1998, Parillo et al., 1992, Bhaswant et al., 2015). In addition, we used glucose as a source of carbohydrate and it is well established that type of carbohydrate influences insulin levels (Augustin et al., 2002, Jenkins et al., 2008). Although we cannot exclude the possibility that fat ingestion may alter the hepatic extraction of insulin, the simplest explanation would invoke the potentiation of insulin secretion by the co-ingestion of fat (Collier et al., 1984, Estrich et al., 1967). Generally, the impact of carbohydrates on blood glucose is affected by the type of carbohydrate, the food form, type and amount of dietary fibre (Guillon and Champ, 2000, Eastwood and Morris, 1992, Granfeldt et al., 2008), the presence of other nutrients.

Lipaemic response

Despite the insulin response being similar in the glucose and combined trials, effects on NEFA suppression differed markedly. There was a substantial initial suppression of NEFA in the glucose trial which was markedly attenuated when glucose and fat were co-ingested. As it has been well established that the ingestion of carbohydrate

stimulates insulin release, which suppresses the release of NEFA from adipose tissue and stimulates fat storage (Sadur and Eckel, 1982, Coppack et al., 1990). However, TG concentrations did not change when OGTT meal was consumed. As carbohydrate has no influence on plasma TG concentrations.

In the OFTT meal, there was a significant postprandial increase in TG concentrations, in line with previous research which has shown that fat content influences plasma TG concentrations (Cohen and Berger, 1990, van Tol et al., 1998). The amount of fat required to significantly elevate plasma TG concentration is in the order of 30-50 g. Some studies have been performed with increasing doses of dietary fat (Cohen et al., 1988, Dubois et al., 1994, Murphy et al., 1995, Dubois et al., 1998). In these studies a very low dose (5 g) or low dose (15 g) of dietary fat does not significantly increase TG concentration postprandially; moderate doses (30-50 g) dose-dependently increase postprandial triglyceridemia; and finally, very high doses (80 g and above) exaggerate postprandial triglyceridemia.

The co-ingestion of carbohydrate with fat did not influence the postprandial plasma TG response in the present study. Many studies reported that the addition of carbohydrate to a fat meal did not influence the lipaemic response after meal ingestion (Cohen et al., 1988, Nicholls and Cohen, 1985, van Oostrom et al., 2004) but this finding is not universal. On the other hand, other studies report that the addition of carbohydrate to a fat meal reduces the post-prandial TG response to the ingested fat compared with a fat meal alone (Albrink et al., 1958, Cohen and Berger, 1990, Westphal et al., 2002, Westphal et al., 2004). This has been linked to effect of carbohydrate in the delay the appearance of ingested lipid as a result of a change in gastric osmolarity (Cohen and Berger, 1990, Hunt, 1961, Westphal et al., 2002, Vist and Maughan, 1995, Jenkins et al., 1984). It has been found that the rate of gastric emptying is reduced roughly 50% when carbohydrate is added to a fat meal (Westphal et al., 2002). However, our findings disagree with this result. This might be due to the hepatic VLDL and chylomicron remnants accumulation due to altered lipoprotein secretion and/or clearance (Parks et al., 1999, Abbasi et al., 2000). As the current data show, chylomicron-TG concentrations were similar when fat ingested alone and/or when it was combined with carbohydrate. And as we mentioned above, insulin

response did not differ when fat was added to carbohydrate which affected LPL secretion and activity. Insulin has a role in the activity of adipose tissue LPL (Nilsson-Ehle et al., 1975, Sadur and Eckel, 1982, Sadur et al., 1984).

It has been observed that postprandial lipaemia is influenced by the amount and type of dietary fat present in the meal, as well as other dietary components including fibre, glucose, starch, and alcohol (Cohen and Berger, 1990, van Tol et al., 1998). It has been reported that the intake of long-chain omega(n) - 3 polyunsaturated fatty acids (PUFAs) (predominantly fish oil), results in lower TG levels and attenuates postprandial lipaemia (Tinker et al., 1999).

Lipoproteins

TG concentration was mostly constant over the course of the OGTT trial and this might be because there was no competition between chylomicron and VLDL₁ particles to be cleared by the action of LPL (Karpe et al., 1992). This also is similar to the pattern of plasma TG and agrees with the findings of others (Lewis et al., 1993b, Lewis et al., 1993a, Vogelberg et al., 1980).

In OFTT and COMB meal the response of chylomicron and VLDL TG paralleled that of total TG; maximum increases occurred 4 h after intake of the meals, a finding that was also observed by others (Karpe et al., 1995, Cohen et al., 1988). The addition of carbohydrate to fat did influence the composition of VLDL₁ (**Figure 5.10**). When carbohydrate ingested with fat or when it was ingested alone, VLDL₁ concentrations were lower than when fat was ingested alone, and this might be due the accumulation of VLDL₁ particles and increase in the clearance of very rich TG-chylomicrons. Alternatively, it might be due to low insulin concentration and high circulating NEFA concentration which stimulate the production of VLDL particles (Lewis et al., 1994, Lewis et al., 1995). Also, it has been suggested that the increase in the influx of exogenous TRL into plasma will inhibit the clearance from plasma of TRL of endogenous origin, whereas an increase in the secretion of TRL of endogenous origin will inhibit the removal from plasma of newly absorbed TRL of exogenous origin (Schneeman et al., 1993). However, TG concentrations were higher in both trials

significantly when compared with fat ingestion alone. A trend of lower protein content in VLDL₁ was observed. This would be linked to the number of particles and suggest that consuming carbohydrate with fat contributes to lower particle number, but more TG-rich particles. Lipolysis of VLDL₁ has been shown to give rise to LDL particles in the density range that have a prolonged residence time (approx. 5 days), compared with LDL derived from smaller VLDL or intermediate-density lipoprotein precursors (which has a residence time of approx. 2 days) (Packard, 2003, Julius et al., 2007, Gazi et al., 2005). The newly formed LDL particles have sufficient time to be remodelled by the action of CETP, LDL will lose CE and gain TG. When this remodelling of LDL particles occurs to a significant degree, then it is postulated that the next exposure of the triacylglycerol-enriched LDL to HL will lead to the removal of enough TG to promote a shift in particle size into the small, dense range (Packard, 2003, Julius et al., 2007, Gazi et al., 2005). This sdLDL are the most readily oxidized subfraction among lipoprotein classes (Tribble et al., 1994, Julius et al., 2007) which increase its atherogenic potential.

In contrast, VLDL₂ particles seem not to respond to any factors neither diet nor exercise as shown in this study and the study in **Chapter 4**. However, there was a trend of higher concentration of VLDL₂ when fat alone was ingested, following the same pattern of VLDL₁ particles. This mean more VLDL₁ and VLDL₂ were accumulated in the circulation when fat alone was ingested.

When consuming carbohydrate alone, IDL tended to have more TG and less phospholipids when compared to co-ingestion or fat ingestion and this is similar to VLDL₁ pattern during the trials.

In the COMB trial IDL concentrations on the plasma were reduced, consistent with the lower concentrations of VLDL₁ particles recorded in this trial. Also, this suggests a faster catabolism of VLDL₁ particles due to the insulin-induced LPL activity (Nilsson-Ehle et al., 1975, Sadur et al., 1984, Sadur and Eckel, 1982).

Concentration of LDL particles did not differ between the three trials. However, compositions differed. Ingesting carbohydrate alone increased the percentage of TG and lowered the percentage CE in LDL particles when compared to ingesting fat alone and/ or the co-ingestion of both. This might be an adverse consequence as smaller more cholesterol rich LDL particle sizes are statistically correlated with other risk factors for coronary artery disease (Rosenson et al., 2002, Lamarche et al., 2008). Because LDL particle size is inversely related to plasma TG concentration, it is important to address if whether elevations in plasma TG induced by adding carbohydrate to fat is associated with reductions in LDL particle size (Dreon et al., 1994, Dreon et al., 1999). Also, its influence, the prevalence of LDL subclass pattern B, a categorical marker for atherogenic dyslipidemia defined by the predominance of sdLDL, has been linearly and positively associated with increasing concentrations of dietary CHOs in randomized controlled clinical trials (RCTs) (Schwingshackl and Hoffmann, 2013, Bueno et al., 2013, Krauss et al., 2006).

However, one of the limitations in the study is that, it was not possible to determine rates of production and removal of lipoprotein particles; this would require a kinetic study. We didn't measure CETP in plasma, which promotes the triacylglycerol enrichment of HDL with concomitant cholesteryl ester accumulation in VLDLs (Dullaart et al., 1989, Mann et al., 1991). We also did not measure the activity of LPL. Moreover, the types of ingested fatty acid have a different influence on lipoprotein composition. It has been shown that, the long term of ingesting specific fatty acids classes can affect serum total, LDL, and HDL cholesterol concentrations and TG concentrations (Mensink and Katan, 1992, Mensink et al., 2003). The type of fatty acid that ingested in the current study was principally saturated and further study is needed evaluate the effects of co-ingestion of different types of fatty acids with carbohydrate.

The meal we provide contained 2.3 g of protein, which is a low amount. The existence of protein in diet might influence plasma lipid postprandially. High protein diets appear to have beneficial effects on weight loss, body composition, and certain blood lipids, at least in the short term (Hession et al., 2009). Satiating effects of dietary protein, a reduced choice of foods, and an aversion against dietary fat in the absence

of carbohydrates have all been attributed to better weight loss with high protein diets (Weickert et al., 2005). However, there is limited data on the amount of protein that can influence postprandial response (Lopez-Miranda et al., 2007). Previous studies suggest that adding fat and protein to carbohydrate reduces glycaemic responses nonlinearly, with the glycaemic impact reaching a plateau as more and more protein and fat are added (Owen and Wolever, 2003, Moghaddam et al., 2006, Spiller et al., 1987). It has been observed that ingesting 47 g of soya protein led to lower cholesterol in plasma and LDL particles and plasma TG (Anderson et al., 1995). Another study found that consuming 20 g of soya protein for 3 weeks rescued plasma remnant like (Higashi et al., 2001). Further investigation should be focused on the effect of ingesting protein alone or with other macronutrients on postprandial metabolism.

The challenges that accompany scientific research in this area result in an inability to conclusively determine the most effective macronutrient compositions required for the reduction of CVD risk. The co-ingestion of both carbohydrate and fat seems to reduce glycaemic response, however, ingesting fat contributes to weight gain in the long term (Lissner and Heitmann, 1995, McMorrow et al., 2016, Hall et al., 2015, Purnell et al., 1999, Visscher and Seidell, 2001, Seidell, 1998, Bray and Popkin, 1998).

6 A pilot study to determine the effect of ethnicity on postprandial metabolism: a comparison between men of European and Middle-Eastern origin

6.1 Introduction

Cardiovascular disease is major cause of morbidity and mortality worldwide, and its incidence is increasing in developing countries, including those in the Middle East, such as Afghanistan, Turkey, Iran, Iraq, Lebanon, Gulf countries, and Palestine (Bovet et al., 2006). Indeed, approximately 80% of the CVD mortality worldwide occurs in developing countries (Sibai et al., 2010). These numbers are expected to continue to rise over coming years and CVD is also more likely to occur in younger ages in developing countries (Bovet et al., 2006). On the other hand, in the Western countries where the disease first had its epidemic, there is now a decline in CVD because of major public health efforts (Mensah et al., 2017, Mendis, 2017).

The pattern of increasing disease burden in the developing countries is not only the case for CVD, but other risk factors and metabolic diseases. For example, in Middle East, overall prevalence of diabetes amongst adults aged 18-80 years is 22% in men and 19% in women (Shara, 2010), and over the last 3 decades obesity in some regions of the Middle East has tripled (Shara, 2010). The Middle East is now facing an epidemic of diabetes and obesity, with prevalence of type 2 diabetes amongst the highest in the world.

Changes in lifestyle factors, including changes to eating habits to more closely resemble Western societies, smoking, urbanization and technological advances and reduced physical activity during work and leisure are likely to contribute to the increases in cardio-metabolic disease in the Middle East (Al-Kandari, 2006). While modifiable lifestyle-related factors are likely to underpin these disease increases (Jamrozik et al., 2001), it is possible that the extent to which these factors influence

disease risk is influenced by unmodifiable risk factors such as age, sex and ethnic background.

There is evidence of ethnic differences in postprandial responses to meal ingestion which could potentially contribute to the observed ethnic differences in cardio-metabolic disease risk (Mohan et al., 1986, Raji et al., 2001). For example, young adults of Vietnamese, south Asian and Chinese origin displayed marked postprandial hyperglycaemia and hyperinsulinemia compared with age-matched Caucasian subjects (Dickinson et al., 2002). Furthermore, in South Asians, the glycaemic response was greater than in Northern Europeans and Latin Americans, although there were no differences in postprandial TG concentrations or in insulin sensitivity as assessed with the insulin tolerance test (Cruz et al., 2001a, McKeigue et al., 1991). Remnant-like particle cholesterol levels in Japanese subjects have consistently been reported to be lower than those in Caucasians (Twickler et al., 2004). Black populations, are more insulin resistant than white Europeans and have higher glucose and insulin responses to glucose ingestion (Healy et al., 2015, Ziemer et al., 2010, Osei et al., 1992).

However, there are limited data on the metabolic responses of Middle Eastern populations, who are a group with high population burden of cardio-metabolic disease. Therefore, this experimental chapter is a pilot study to examine the differences in postprandial metabolic responses between Middle Eastern and European adults.

6.2 Participants and Methods

6.2.1 Participants

The data of 16 men (8 Middle Eastern and 8 Europeans) were collected from the previous experimental Chapter 4 and 5, their characterizations are listed in Table 6.1. All subjects were apparently healthy, normotensive, normoglycaemic (fasting

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glucose ≤ 6 mmol.l⁻¹), nonsmokers. None was taking any drugs known to affect lipid or carbohydrate metabolism. The study was conducted with the approval of University of Glasgow Ethics Committee, and subjects gave written informed consent prior to participation.

6.2.2 Test meals

Seven participants had the high fat mixed meal (HFM) meal (containing 75 g fat and 77.4 g carbohydrate) described in **section 2.5.1** and 9 participants had the COMB meal (containing 92.4 g fat and 86.3 g carbohydrate) described in **section 2.5.4** (see **table 6.1**). Test meal was included as a co-variate in statistical analyses and shown not to have a significant effect on any of the postprandial responses.

6.2.3 Blood sampling

Subjects reported to the lab on the morning after fasting for at least 12h. A cannula was placed in an antecubital vein and, after a 10-min interval, a fasted state blood sample was withdrawn. A test meal, as described above in **Section 6.2.2.**, was provided. Further blood samples were obtained at 30, 60, 90, 120 and 240 minutes after meal consumption. Subjects rested and consumed only water during this time.

Table 6.1; Physical characteristics

	Meal	AGE (years)	Height (M)	Body Mass (Kg)	BMI (kg.m ⁻²)	Waist (cm)	Hip (cm)	WHR	% body fat
European	HFM	37.00	1.91	101.90	27.93	102.00	107.00	0.95	23.31
	HFM	24.00	1.68	77.20	27.29	85.00	95.10	0.89	16.79
	HFM	34.00	1.69	75.40	26.28	94.05	99.45	0.95	26.27
	HFM	49.00	1.71	81.00	27.67	101.00	103.00	0.98	25.55
	COMB	32.00	1.85	86.35	25.07	88.81	102.10	0.87	24.74
	COMB	31.00	1.75	83.00	27.10	79.85	98.45	0.81	25.93
	COMB	25.00	1.84	83.10	24.52	95.00	102.00	0.93	15.09
	COMB	35.00	1.85	86.00	25.13	81.15	102.70	0.79	21.57
Mean SD		33.38	1.79	84.24	26.37	90.86	101.23	0.90	22.41
		7.80	0.09	8.11	1.32	8.52	3.55	0.07	4.30
Middle Eastern	HFM	55.0	1.8	89.60	28.83	101.60	103.50	0.98	33.00
	HFM	31.0	1.7	81.00	27.38	102.00	98.00	1.04	27.00
	HFM	35.0	1.8	83.00	27.10	95.50	102.00	0.94	25.93
	COMB	32.0	1.7	101.30	33.54	102.00	111.00	0.94	28.48
	COMB	35.0	2.0	90.00	22.96	74.00	98.00	0.76	10.56
	COMB	29.0	1.8	104.00	32.82	109.50	119.30	0.92	26.46
	COMB	29.0	1.9	94.00	27.47	95.50	100.00	0.96	27.00
	COMB	25.0	1.8	82.00	24.49	78.00	98.00	0.80	11.35
Mean SD		33.88	1.80	90.61	28.07	94.76	103.73	0.91	23.73
		9.16	0.08	8.71	3.66	12.44	7.66	0.09	8.16
p-value (EU vs ME)		0.91	0.74	0.15	0.24	0.48	0.42	0.71	0.69

Values are means (SD), EU = European, ME = Middle Eastern. N= 8 European and 8 Middle eastern.

6.2.4 Plasma assays

Serum, TG and NEFA and plasma glucose concentrations were analysed at all-time points as previously mentioned in **Appendix C**. Total, HDL cholesterol and sdLDL concentrations were measured in the fasted state as mentioned in **Appendix C**. LDL cholesterol was calculated in the fasted state using the Friedewald equation (Friedewald et al., 1972). Insulin was measured in EDTA plasma, as described in **section 2.6.3**, HOMA-IR was calculated as described in **section 2.6.4**.

6.2.5 Lipoprotein separation

Plasma samples (2 ml) at 0, 1, 2 and 4 hours were centrifuged using ultracentrifugation technique to isolate lipoprotein subfractions as previously described in **section 2.6.2**. VLDL₁ and VLDL₂ fractions were assayed to determine concentrations and composition for TG, free cholesterol (FC) and phospholipids, as mention in **Appendix C** Cholesterol Ester was determined as described in section. Chylomicron fractions were assayed for TG concentration. In the VLDL₁ and VLDL₂ fractions, total protein was measured using a modified Lowry assay (see **section 2.7**).

6.2.6 Calculations and statistical analysis

Statistical analyses were performed using Statistica (version 10, StatSoft Inc.) and Minitab (version 17, Minitab Ltd). All data were tested for normality using the Anderson-Darling test. Where data did not approximate a normal distribution, these were log-transformed prior to analysis. Time-averaged postprandial concentrations, calculated as the trapezium rule-derived areas under concentration versus time

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curve, divided by the duration of the postprandial observation period (240 minutes), were used as summary measures of the postprandial responses. Postprandial concentrations at individual timepoints between the two ethnic groups were compared by repeated measures ANOVA, with adjustment for BMI, age and test meal (HFM vs COMB). *Post hoc* Fisher tests were carried out to establish where differences lay. When displayed graphically SEMs were used as error bars. Unpaired T-tests were used to calculate differences in the area under the curve and the incremental area under the curve for postprandial responses. Statistical significance was set at $p < 0.05$. Cohen's d effect size was calculated by dividing the mean difference between the two groups by the standard deviation pool of both groups. Percentages of TG and CE were relative to total mass. TG to CE ratio in lipoprotein fractions was calculated as: TG/CE.

6.3 Results

6.3.1 Fasting plasma concentration

Table 6.2. shows fasting plasma concentrations for both groups. European had higher fasting TG concentration than Middle Eastern by 40% ($p = 0.05$), with a large Cohen's d effect size for this difference of 0.96. There were no significant differences in total cholesterol, HDL, sdLDL, NEFA, glucose or insulin concentrations. However, although the differences did not achieve statistical significant fasting insulin concentrations and HOMA-IR were both ~4 fold higher in the Middle Eastern compared with the European adults, with medium Cohen's d effect sizes of 0.71 and 0.68 respectively.

Table 6.2; Fasting Plasma Values.

	European (n = 8)	Middle Eastern (n = 8)	Mean difference	Effect size	P- value
Total cholesterol (mmol.l ⁻¹)	5.13 ± 0.36	4.91 ± 0.36	+0.23	0.23	0.66
HDL (mmol.l ⁻¹)	1.20 ± 0.09	1.35 ± 0.17	-0.15	0.39	0.46
SdLDL (mg.dl ⁻¹)	35.56 ± 6.58	28.50 ± 5.03	+7.06	0.43	0.41
Triglyceride (mmol.l ⁻¹)	1.32 ± 0.17	0.88 ± 0.12	+0.44	0.96	0.05
NEFA (mmol.l ⁻¹)	0.75 ± 0.06	0.79 ± 0.08	-0.04	0.22	0.68
Glucose (mmol.l ⁻¹)	5.00 ± 0.19	5.11 ± 0.18	-0.10	0.20	0.70
Insulin (mU.l ⁻¹)	5.81 ± 0.76	22.16 ± 11.07	-16.36	0.71	0.16
Log-Insulin (mU.l ⁻¹)*	1.70 ± 0.14	2.39 ± 0.44	-0.69	0.72	0.16
HOMA-IR (units)*	1.30 ± 0.20	5.07 ± 2.70	-3.77	0.68	0.19

Values are means ± SEM, *log transformed values, *Homeostasis Model Assessment. . Statistical analysis performed using unpaired t-test.

6.3.2 Postprandial concentrations

Table 6.3 shows the summary postprandial responses for the two ethnic groups. Europeans had higher TG AUC by 42%; this had borderline statistical significance ($p=0.057$) and a large Cohen's d effect size. Figure 6.1 shows these responses graphically; TG concentrations were significantly higher in Europeans at both the 2 and 4 hour postprandial timepoints. A similar pattern was observed when considering the IAUC and postprandial rises in TG concentration (i.e. adjusting for the difference in the fasting TG concentration). The TG IAUC was 62% higher in the Europeans, this difference was not statistically significant ($p = 0.13$), but the Cohen's d effect size, at 0.77 was moderate to large. The rise in postprandial TG concentration above fasting was significantly higher in the Europeans than the Middle Eastern participants at 2 and 4 hour postprandial timepoints (see Figure 6.1). The difference between the two groups is clear at 2 and 4 hours postprandially. At 2 and 4 hours postprandially TG concentrations differ between the two groups significantly, as European shows higher TG response by 42% and a large Cohen d effect size 0.92 ($p=0.01$) and 50% with a large Cohen d effect size of 0.94 from Middle Eastern ($p=0.001$) respectively. The same trend was observed in the incremental area under the curve at 2 and 4 hours, European TG was 46% higher ($p=0.03$) to give a medium Cohen's d effect size 0.76 at 2 hours postprandially and 60% higher at 4 hours postprandially ($p=0.005$) and an effect size of 0.82 (Figure 6.1 panel B).

There was no significant difference in AUC and IAUC for log-transformed glucose, log-transformed insulin or NEFA (Table 6.3, Figure 6.2 panel A and B, Figure 6.3 panel A and B and Figure 6.4 panel A and B). However, both glucose and insulin

concentrations were different significantly in single time points between the two groups. Plasma glucose values at 60 and 90 minutes after meal ingestion were lower in European by 24% giving a large effect size of 0.90 ($p=0.001$) and 29% with a large effect size of 0.85 ($p=0.05$) respectively when compared with Middle Eastern. Also, incremental plasma glucose values at 60 and 90 minutes after meal ingestion were lower in European by 188% ($p=0.005$) and 27% ($p=0.05$) and to give a large Cohen's d effect size of 0.91 and 0.94 respectively comparing with Middle Eastern (**Figure 6.2 panel B**).

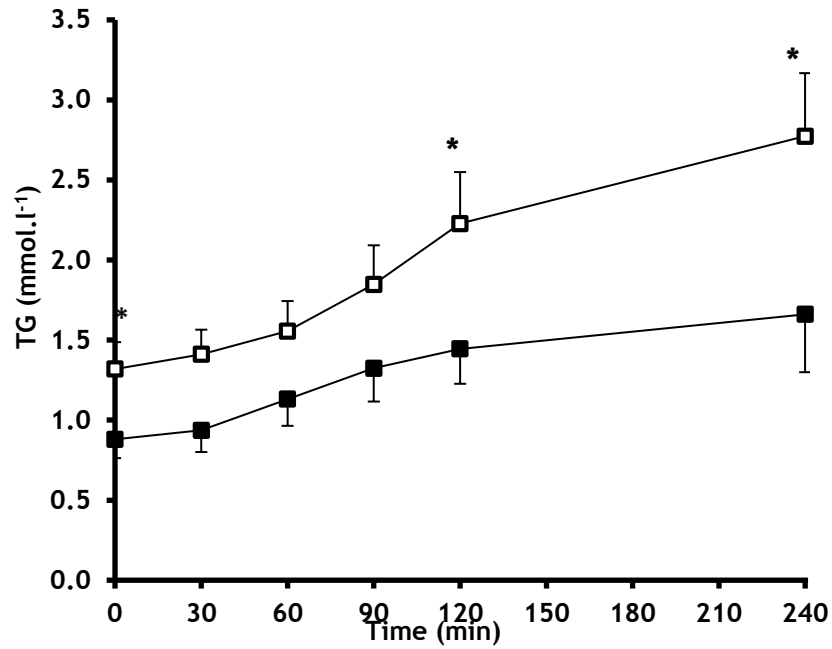
Similarly insulin values were significantly lower in European compared with Middle Eastern, at 90 and 120 minutes was higher by 128% and 88% respectively compared with European ($p=0.02, p=0.05$) giving a Cohen's d effect size of 0.94 and 0.66 respectively. IAUC was higher in Middle Eastern at 90 minutes by 129% compared with European ($p=0.005$) with a large Cohen's d effect size 0.88 (see **Figure 6.3 panel B**).

Table 6.3; Time-Averaged and incremental postprandial plasma Concentration.

		European (n= 8)	Middle eastern (n=8)	Mean difference	Effect size	P-value
AUC	Triglyceride (mmol.l ⁻¹)	2.07 ± 0.27	1.35 ± 0.22	+0.73	0.94	0.057
	NEFA (mmol.l ⁻¹)	0.80 ± 0.08	0.81 ± 0.08	-0.01	0.05	0.92
	Log-Glucose (mmol.l ⁻¹)*	1.59 ± 0.04	1.71 ± 0.06	-0.12	0.83	0.10
	Log-Insulin (mU.l ⁻¹)*	2.98 ± 0.15	3.54 ± 0.42	-0.56	0.61	0.24
IAUC	Triglyceride (mmol.l ⁻¹)	0.76 ± 0.14	0.47 ± 0.11	+0.29	0.77	0.13
	NEFA (mmol.l ⁻¹)	0.05 ± 0.05	0.04 ± 0.07	+0.01	0.05	0.92
	Glucose (mmol.l ⁻¹)	0.06 ± 0.14	0.51 ± 0.25	-0.57	0.90	0.07
	Insulin (mU.l ⁻¹)	15.69 ± 3.84	37.83 ± 16.58	-22.14	0.64	0.21

Values are mean time-averaged postprandial concentration ± SEM, and mean incremental rise in postprandial state ± SEM, N= 8 European and 8 Middle eastern.*log-transformed data. Statistical analysis performed using unpaired t-test.

[A] Mean postprandial plasma TG



[B] Incremental time-averaged plasma TG

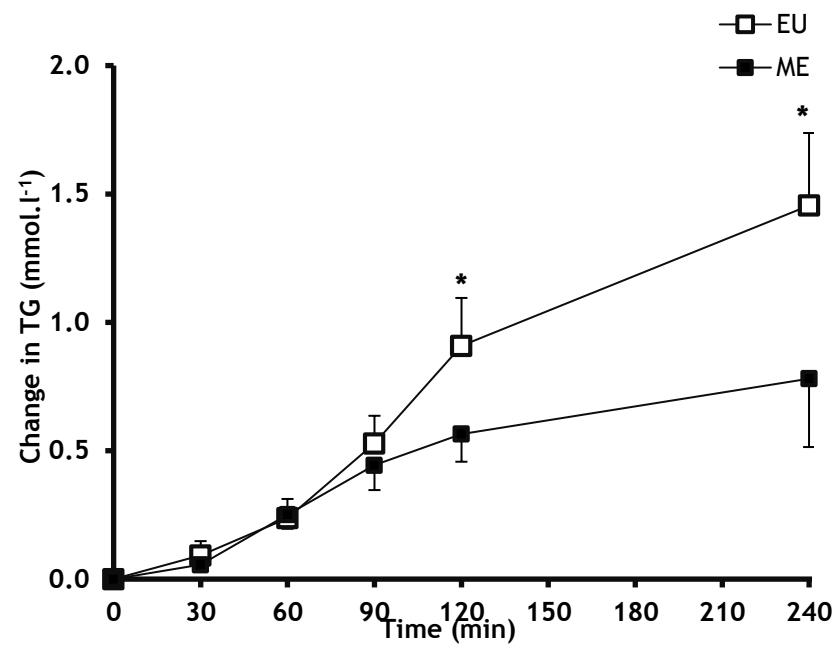


Figure 6.1. Plasma-TG concentrations during 4 h observation period in both groups; European and Middle Eastern. Panel [A] shows the mean values for TG concentrations; panel [B] shows the incremental time-averaged area under the curve for plasma TG, N= 8 European and 8 Middle eastern, Values are mean \pm SEM. * significant values from the other group ($p < 0.005$), ** ($p \leq 0.05$). Values and statistical analysis of these data is shown in Table 6.3.

[A] Mean postprandial plasma glucose

[B] Incremental time-averaged plasma glucose

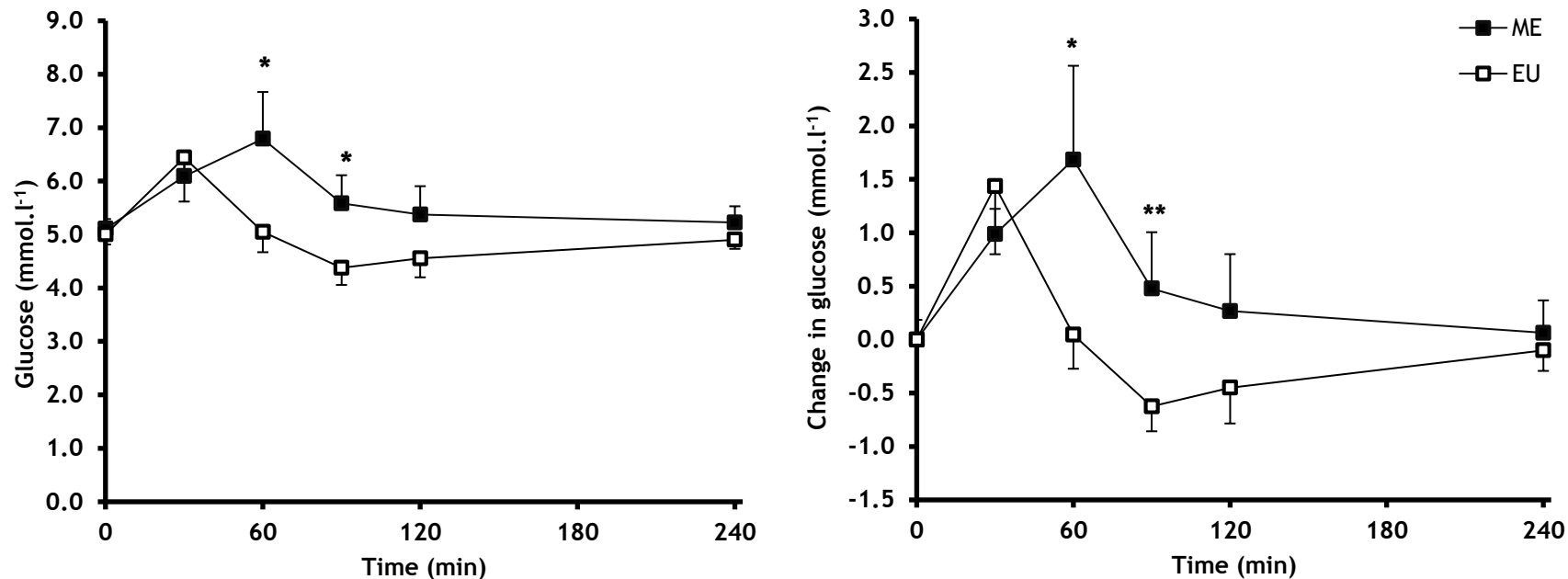


Figure 6.2. Plasma-glucose concentrations during 4 h observation period in both groups; European and Middle Eastern. Panel [A] shows the mean values for glucose concentrations; panel [B] shows the incremental time-averaged area under the curve for plasma glucose. N= 8 European and 8 Middle eastern, Values are mean \pm SEM. * significant values from the other group ($p < 0.005$), ** ($p \leq 0.05$). Values and statistical analysis of these data is shown in Table 6.3.

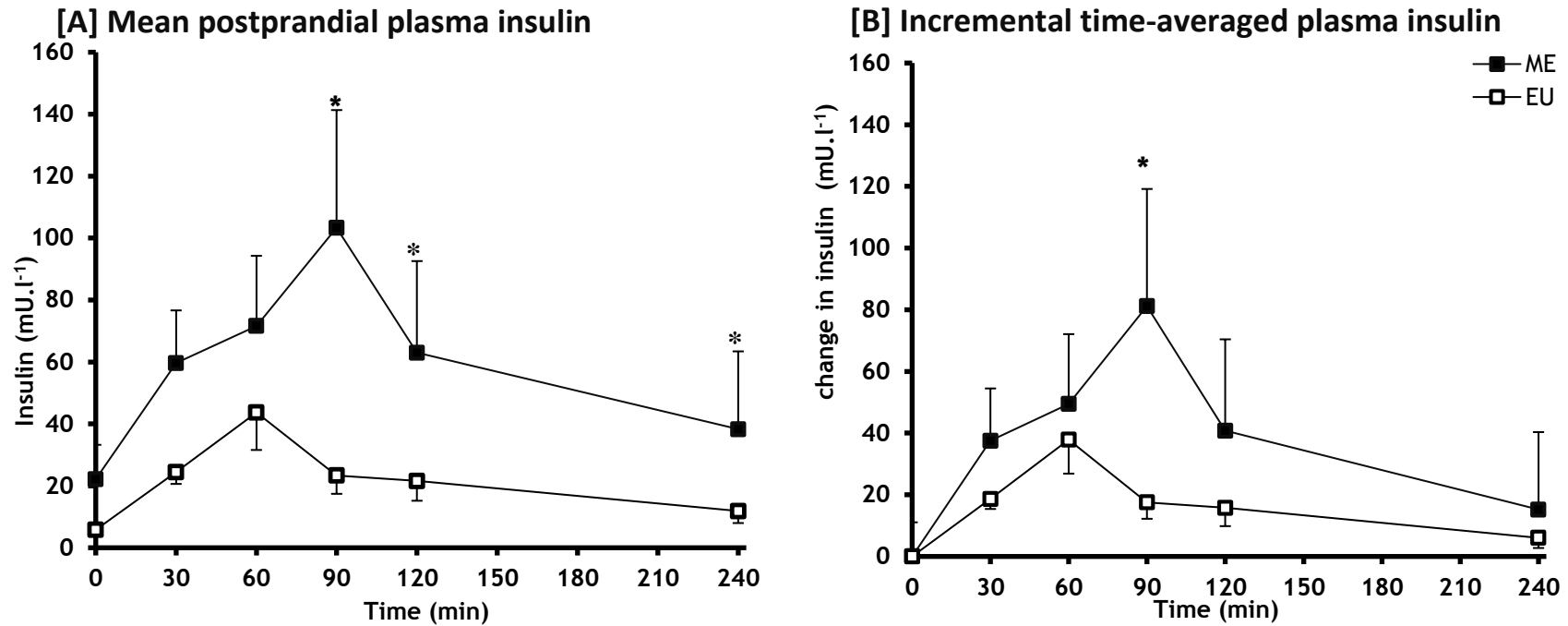


Figure 6.3 . Plasma-insulin concentrations during 4 h observation period in both groups; European and Middle Eastern. Panel [A] shows the mean values for insulin concentrations; panel [B] shows the incremental time-averaged area under the curve for plasma insulin. N= 8 European and 8 Middle eastern, Values are mean \pm SEM. * significant values from the other group ($p < 0.005$), ** ($p \leq 0.05$). Values and statistical analysis of these data is shown in Table 6.3.

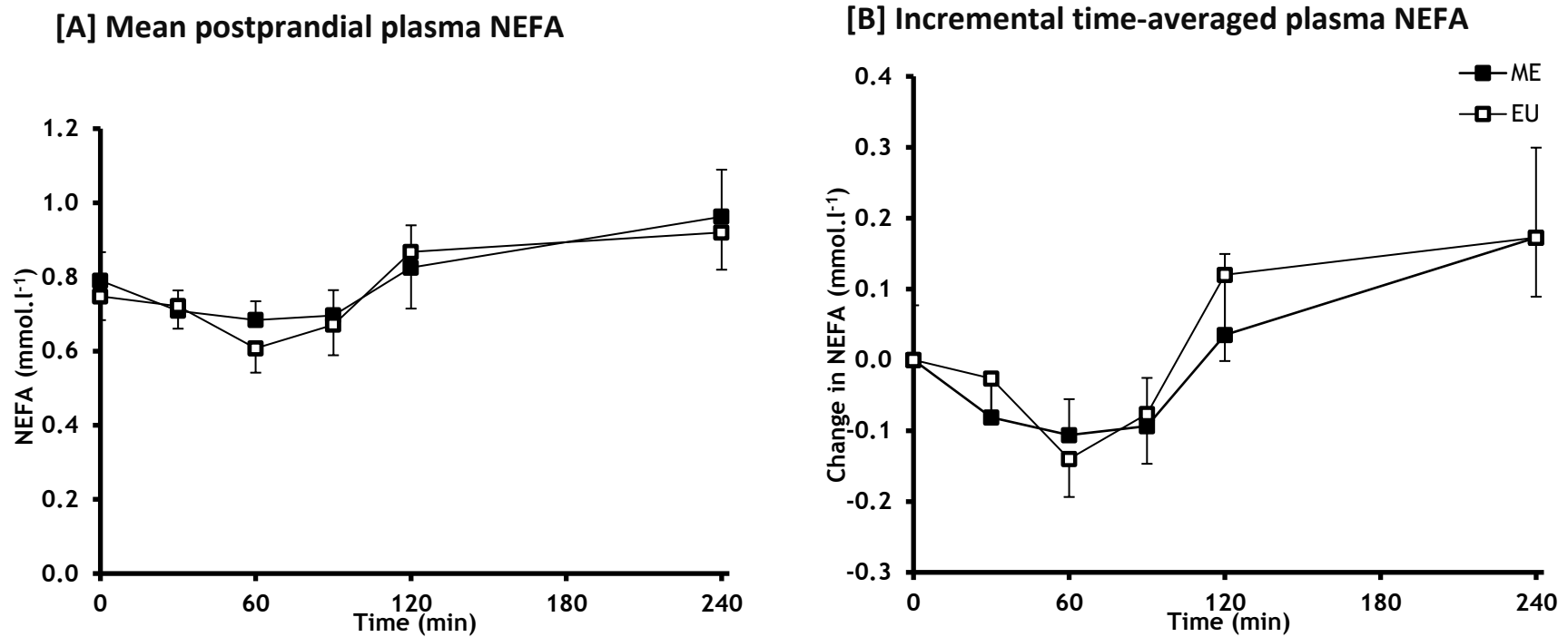


Figure 6.4. Plasma-NEFA concentrations during 4 h observation period in both groups; European and Middle Eastern. Panel [A] shows the mean values for NEFA concentrations; panel [B] shows the incremental time-averaged area under the curve for plasma NEFA. N= 8 European and 8 Middle eastern, Values are mean \pm SEM. * significant values from the other group ($p = 0.005$). Values and statistical analysis of these data is shown in Table 6.3.

6.3.3 Lipoprotein subfractions

Fasted Lipoprotein concentrations

Fasted VLDL₁ and VLDL₂ composition show in **Table 6.4**. VLDL₁ concentrations were significantly higher in European by 72% when compared with Middle Eastern ($p < 0.005$), giving a large Cohen's d effect size of 0.96 for this difference (**Figure 6.5 panel A**). There were no other significant differences in VLDL₁ and VLDL₂ percentage of TG, FC, EC, PL, protein or the ratio of TG/CE.

Table 6.4; Fasted values for VLDL₁ and VLDL₂ composition.

		European (n=8)	Middle eastern (n=8)	Mean difference	Effect size	p-value
VLDL ₁	Total lipoprotein concentration (mg.dl ⁻¹)	72.04 ± 15.80	33.55 ± 8.47	+38.49	0.96	0.05
	Triglyceride (%)	60.03 ± 1.84	58.99 ± 2.77	+1.04	0.16	0.76
	Free cholesteryl (%)	7.03 ± 1.17	5.51 ± 0.82	+1.52	0.53	0.31
	Cholesterol ester (%)	9.03 ± 2.08	9.28 ± 1.55	-0.25	0.05	0.92
	Phospholipid (%)	15.12 ± 1.07	14.47 ± 1.30	+0.56	0.20	0.71
	Protein (%)	8.78 ± 1.29	11.73 ± 2.65	-2.95	0.50	0.33
	CE/TG ratio (mol:mol)	0.21 ± 0.05	0.23 ± 0.06	-0.02	0.12	0.82
VLDL ₂	Total lipoprotein concentration (mg.dl ⁻¹)	41.20 ± 8.39	32.33 ± 9.42	+8.87	0.33	0.52
	Triglyceride (%)	34.32 ± 3.82	33.23 ± 4.27	+1.09	0.10	0.85
	Free cholesteryl (%)	9.66 ± 0.71	8.79 ± 0.70	+0.87	0.44	0.40
	Cholesterol ester (%)	22.33 ± 2.18	22.45 ± 2.95	-0.12	0.02	0.97
	Phospholipid (%)	19.99 ± 1.35	18.83 ± 1.82	+1.15	0.26	0.62
	Protein (%)	13.70 ± 2.77	16.70 ± 3.43	-2.99	0.35	0.51
	CE/TG ratio (mol:mol)	1.42 ± 0.36	1.45 ± 0.30	-0.02	0.03	0.96

Values are mean ± SEM.

Postprandial lipoprotein concentrations

Table 6.5 shows chylomicron, VLDL₁ and VLDL₂ concentration in the postprandial state.

There was no significant difference in the postprandial chylomicron-TG concentration (Table 6.5 and Figure 6.5).

VLDL₁ concentration AUC tends to be higher in Europeans when compared with Middle Eastern by 52% ($p=0.09$), giving a large effect size of 0.84. The concentration of VLDL₁ at 2 and 4 hours postprandially rose significantly among European by 58% and giving a large effect size of 0.93 and 42% giving a medium effect size of 0.66, ($p<0.005$ for both) respectively. (Figure 6.5 panel A). There was no significant difference in VLDL₁ concentration IAUC (Figure 6.5 panel B).

VLDL₂ concentration at 2 hours postprandially was significantly higher among European by 43% and giving a medium effect size of 0.75 ($p<0.005$). (Figure 6.7, panel A and B).

There were no significant differences between the two groups in the postprandial AUC or IAUC for both VLDL₁ and VLDL₂ percentage of TG, FC, EC, PL, protein or the ratio of TG/CE. (Table 6.5).

Table 6.5; Time-averaged area under the curve for VLDL₁ and VLDL₂ composition.

		European (n=8)	Middle eastern (n=8)	Mean difference	Effect size	p-value
<i>Chylomicron</i>	Triglyceride (mmol.l ⁻¹)*	1.17 ± 0.27	1.53 ± 0.20	+0.36	0.54	0.30
<i>VLDL₁</i>	Total lipoprotein concentration (mg.dl ⁻¹)	90.42 ± 16.15	53.00 ± 13.20	+37.43	0.84	0.09
	Triglyceride (%)	61.18 ± 1.52	60.92 ± 1.85	+0.25	0.05	0.92
	Free cholesteryl (%)	5.74 ± 0.43	5.09 ± 0.52	+0.56	0.49	0.35
	Cholesterol ester (%)	9.43 ± 0.91	9.60 ± 1.67	-0.17	0.04	0.93
	Phospholipid (%)	15.42 ± 1.02	15.33 ± 1.03	+0.09	0.03	0.95
	Protein (%)	8.23 ± 1.07	9.06 ± 1.18	-0.83	0.41	0.61
	CE/TG ratio (mol:mol)	0.22 ± 0.03	0.27 ± 0.09	-0.05	0.31	0.55
<i>VLDL₂</i>	Total lipoprotein concentration (mg.dl ⁻¹)	42.25 ± 7.24	28.97 ± 5.95	+13.28	0.69	0.18
	Triglyceride (%)	37.23 ± 4.05	33.19 ± 3.41	+4.04	0.39	0.46
	Free cholesteryl (%)	8.35 ± 0.48	8.59 ± 0.55	-0.24	0.17	0.75
	Cholesterol ester (%)	21.05 ± 2.49	21.70 ± 2.72	-0.65	0.09	0.86
	Phospholipid (%)	19.22 ± 1.38	18.95 ± 1.78	+0.27	0.06	0.91
	Protein (%)	14.14 ± 2.73	17.56 ± 2.87	-3.42	0.43	0.40
	CE/TG ratio (mol:mol)	1.41 ± 0.58	1.15 ± 0.32	+0.25	0.20	0.71

Values are AUC mean ± SEM, CM=chylomicron. *log-transformed data.

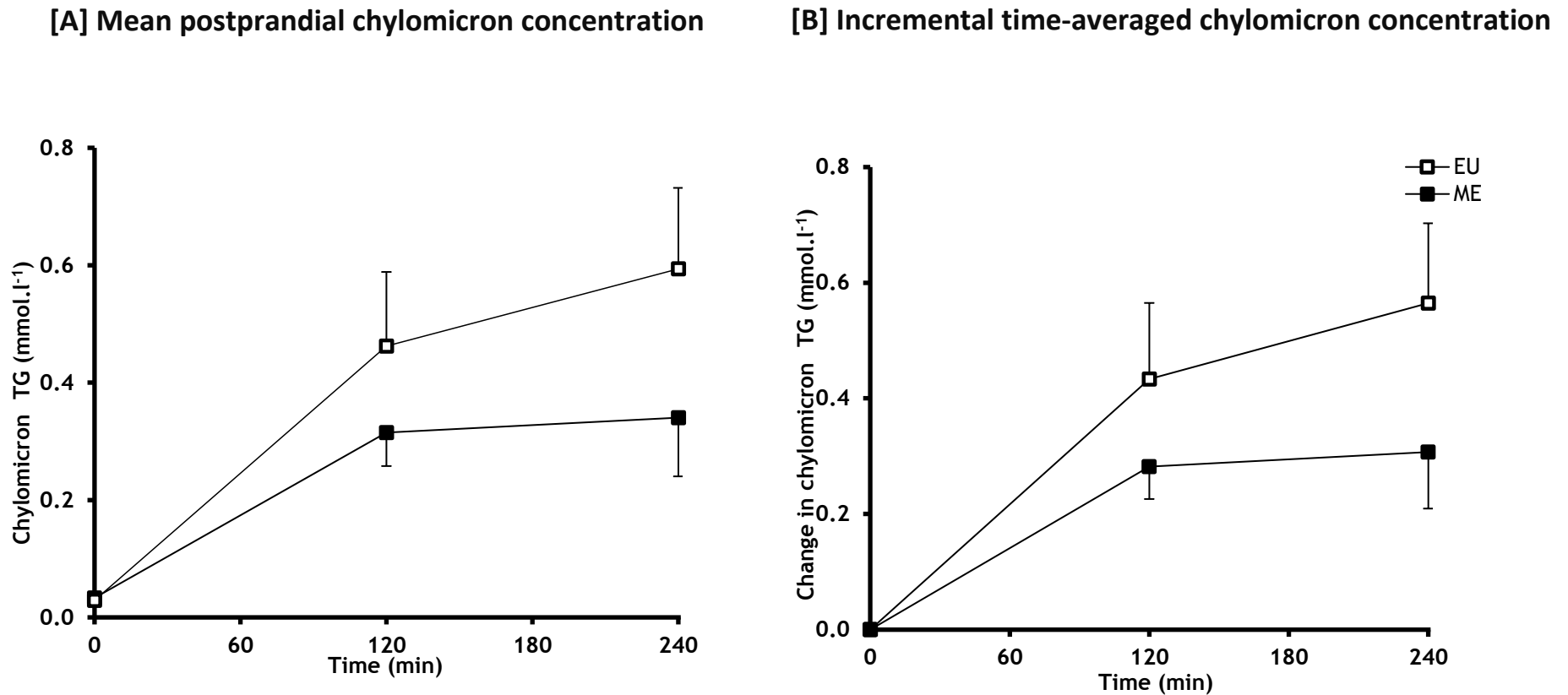
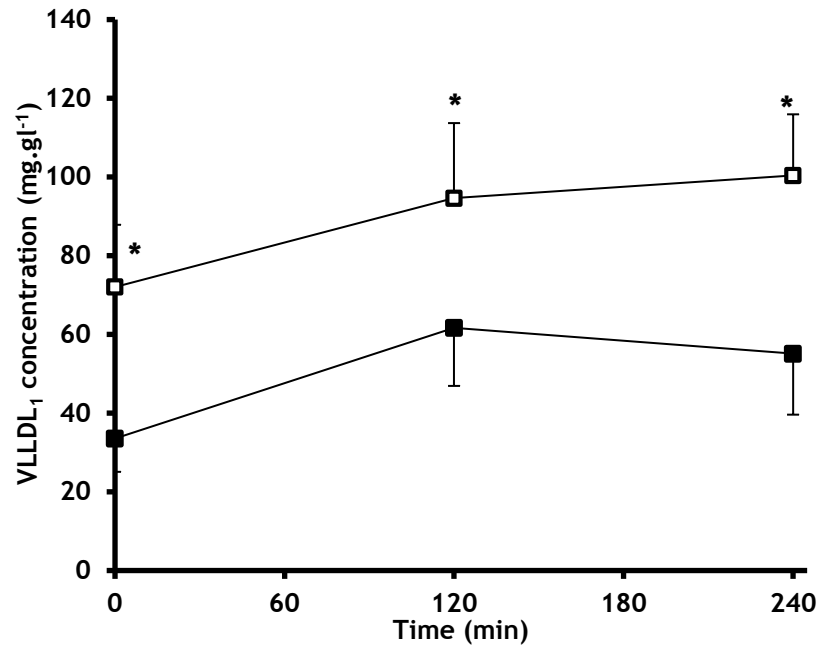


Figure 6.5. Chylomicron concentrations during 4 h observation period in both groups; European and Middle Eastern. Panel [A] shows the mean values for chylomicron concentrations ; panel [B] shows the incremental time-averaged area under the curve for chylomicron concentrations. N= 8 European and 8 Middle eastern, Values are mean \pm SEM. * significant values from the other group ($p < 0.005$). Values and statistical analysis of these data is shown in Table 6.5.

[A] Mean postprandial VLDL₁ concentration



[B] Incremental time-averaged VLDL₁ concentration

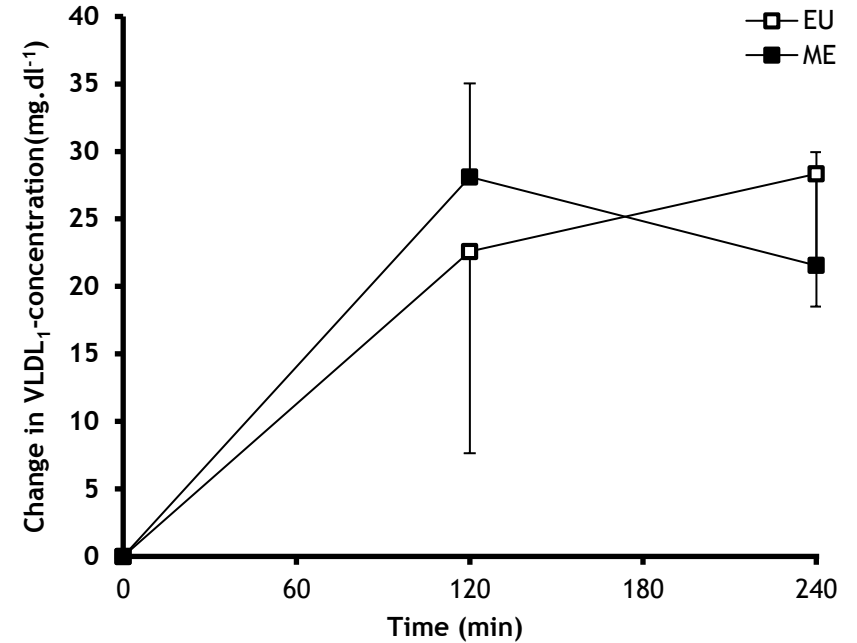
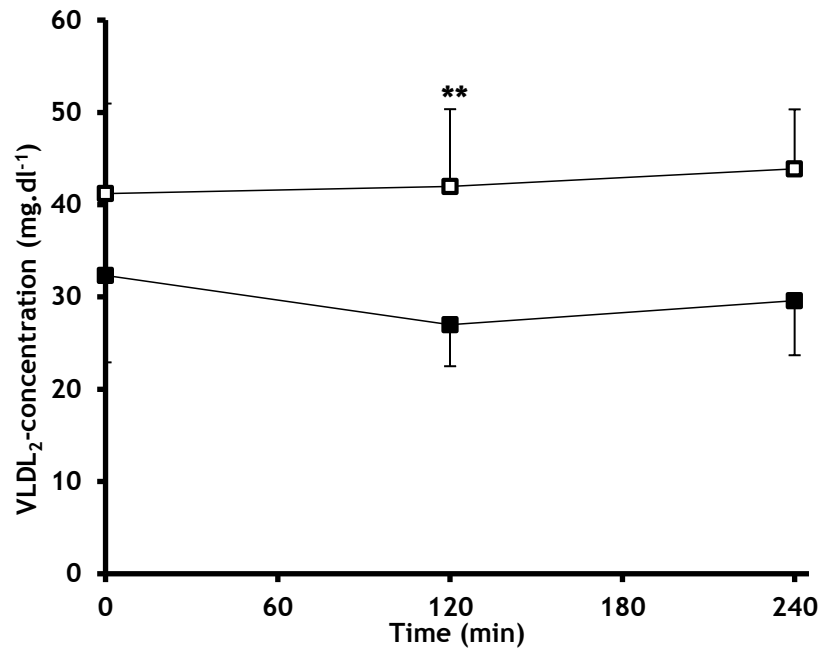


Figure 6.6. VLDL₁ concentrations during 4 h observation period in both groups; European and Middle Eastern. Panel [A] shows the mean values for VLDL₁ concentrations; panel [B] shows the incremental time-averaged area under the curve for VLDL₁ concentrations. N= 8 European and 8 Middle eastern, Values are mean \pm SEM. *significant values from the other group ($p < 0.005$), ** ($p \leq 0.05$). Values and statistical analysis of these data is shown in Table 6.5.

[A] Mean postprandial VLDL₂ concentration



[B] Incremental time-averaged VLDL₂ concentration

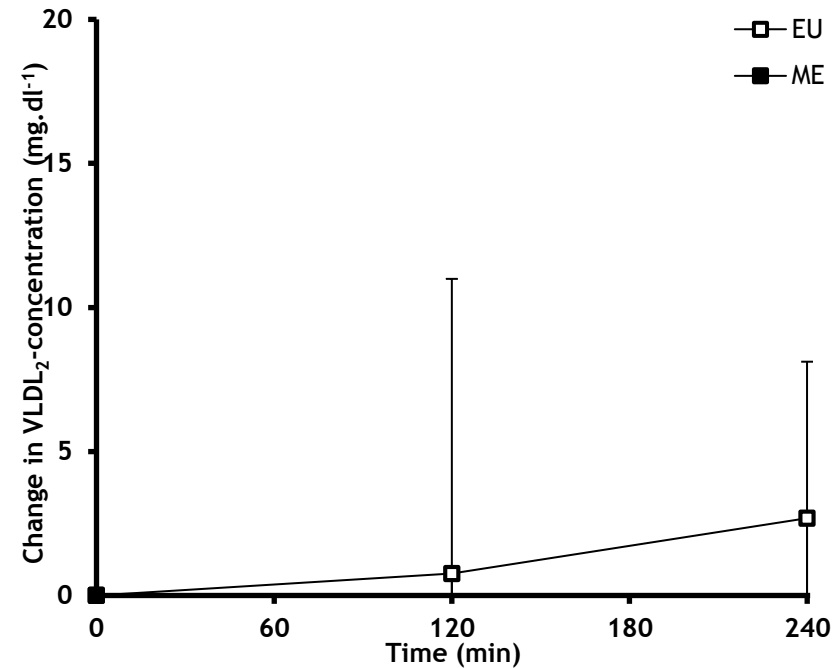


Figure 6.7. VLDL₂ concentrations during 4 h observation period in o in both groups; European and Middle Eastern. Panel [A] shows the mean values for VLDL₂ concentrations; panel [B] shows the incremental time-averaged area under the curve for VLDL₂ concentrations. N= 8 European and 8 Middle eastern, Values are mean \pm SEM. *significant values from the other group ($p < 0.005$), ** ($p \leq 0.05$). Values and statistical analysis of these data is shown in Table 6.5.

6.4 Discussion

The main objective of the present study was to verify whether differences in the postprandial response to a fat challenge between Middle Eastern and European. To provide enough subjects in this exploratory analysis two test meals were included, COMB from **Chapter 5** and the high fat meal from **Chapter 4**. Both contained approximately the same amount of fat although this was presented in a different format. The nature of the test meal was included as a potentially confounding variable in the statistical analysis.

The main findings of this study were that in matched age and BMI overweight European and Middle Eastern men, consuming a mixed test meal the Europeans had higher TG response whereas the Middle Eastern men had the higher insulin response. This profile of the Middle Eastern postprandial response seems to be broadly comparable to what has been observed when comparing Black with White adults (Osei and Schuster, 1994, Kodama et al., 2013). Black adults also have lower TG responses and higher insulin responses than White Europeans (Sumner and Cowie, 2008, Ford et al., 2002). This is interesting because the higher TG levels in plasma are usually thought to be indicative of insulin resistance (Axelsen et al., 1999, Grundy, 1999, Högl et al., 1998, Lovejoy et al., 1996, Albu et al., 1997, Schmidt et al., 1996). A cursory comparison of CVD risk factors in the UK shows striking similarities between African Caribbeans and South Asians, with an increased risk of glucose intolerance and diabetes, and raised fasting and post-load insulin, indicating a greater degree of insulin resistance (Chaturvedi et al., 1993). However, VLDL, small dense LDL, and triglyceride concentrations are substantially lower in African Caribbeans than in Europeans, and the latter does not increase to the same extent in the presence of glucose intolerance (Chaturvedi et al., 1994). In contrast South Asian who have an increased risks of heart disease compared to Europeans, probably because of increased levels of insulin resistance and associated factors such as inflammation and endothelial dysfunction (Chaturvedi, 2003).

In the current study fasted TG concentrations were different and therefore, the postprandial response would be expected to follow the same pattern. It is well

established that, fasting levels of plasma TG tend to be correlated with the magnitude of postprandial lipaemic response (Cohn et al., 1988b). This can be observed in the current study, the lower fasted TG in Middle Eastern men probably led to a lower postprandial response. However, the chylomicron concentrations postprandially shows that there was no significant difference between the two groups and the exact difference is specific to endogenous TG. It has been observed that lean African American women have lower TG response after ingesting high fat meal, comparing with lean Caucasian women this is may be partly due to enhanced expression, activity, and intravascular availability of LPL (Bower et al., 2002). However, the ethnic differences in expression and function of LPL are attenuated with obesity (Bower et al., 2002). Friday and colleagues studied the postprandial response of black and white young men and they found that the black men have lower TG response comparing with whites and this is due to the higher LPL activity (Friday et al., 1999). In the Caucasian population, there is considerable variability in insulin resistance at given levels of body fat content. This variability might be because they tend to secrete a relatively large proportion of their VLDL as small, triglyceride-poor particles, levels of which are not augmented in response to loss of insulin action (Godsland et al., 2007). Another possible mechanism might be due to an increase of the capacity of TG clearance from circulation that is most likely due to a higher LPL activity and LPL mass in adipose tissue, which has been observed previously in Caucasian women compared with African American (Bower et al., 2002). This more efficient lipid-clearing system may help to maintain a more favourable lipid profile and lipoprotein subpopulation distribution. Also, it has been found that, the favourable lipid profile of black compared to white South African women is associated with polymorphisms in lipid metabolism genes, specifically the LPL and CETP genes (Ellman et al., 2015).

Ethnic differences in postprandial glycaemia have been reported previously between Chinese and Europeans (Dickinson et al., 2002), Caucasians and non-Caucasians (Wolever et al., 2009), and Europeans compared with a group of Asians of mixed origin (Schmidt et al., 1996).

Despite there were no significant differences between the two groups in fasted and postprandial insulin and glucose concentrations, there was a trend of higher insulin and glucose concentrations and HOMA-IR (the Cohen's d effect size was

mostly medium) and this is due to the lack of power of the study. The findings suggest that reduced insulin sensitivity and impaired carbohydrate tolerance might lead to the development of some metabolic syndrome. Chronic postprandial hyperglycemia and/or hyperinsulinemia may therefore be the only phenotypic evidence of reduced insulin sensitivity for many years. Abnormalities in fat deposition and blood lipids characteristic of the IR syndrome may develop as a consequence of postprandial hyperglycaemia or hyperinsulinemia. Excessive glycaemia and/or insulin demand on a chronic basis may eventually affect insulin secretory capacity and precipitate T2D. Obesity, particularly visceral adiposity, is associated with insulin resistance and often assumed to be causative. The higher concentrations of fatty acids resulting from higher fat intake or higher visceral or intramuscular fat, inhibit carbohydrate oxidation and thereby produce insulin resistance the present study, however, there was no significant different between the two groups neither in the fat percentage, waist/hip ratio nor BMI. There was no evidence to suggest that there were differences in body fat that might explain our findings. BMI, waist circumference and WHR, did not differ among groups. However, we cannot exclude the possibility that a more precise measurement of body fat using dual X-ray absorptiometry or nuclear magnetic resonance may have shown differences among the ethnic groups.

Regarding the cholesterol measurements, there was no significant different between the two groups in the fasting plasma total, LDL and HDL cholesterol, which is in agreement clinical data showing that Middle Eastern adults tend to have fasting plasma cholesterol level $\sim 5.2 \text{ mmol.l}^{-1}$ - comparable with values in the White UK population. However, the age range in those studies was different then the age range in the current study and there is limited data about the participant BMI (Al-Nozha et al., 2008, Al-Lawati et al., 2003, Zindah et al., 2008).

However, the similar pattern of Middle Eastern to black ethnic group, the Middle Eastern seems to have higher incidence of CVD as 45% of early deaths in the Gulf countries are caused by CVD, whereas black have lower CVD incidence comparing with white ethnic groups (Manolio et al., 1995, Keil et al., 1993). This high incidence of CVD in the Middle Eastern population may be due to the high prevalence of obesity (13% for men and 24.5% for women), diabetes mellitus (11.3%), and smoking (48.04% among men and 13.8% among women) (International

Obesity Task Force 2012, Khattab et al., 2012) in this region. Currently, CVD has emerged as one of the leading causes of death in this population (Tailakh et al., 2014). With one of the highest rates of obesity in the world, the Gulf region is facing an epidemic of cardiovascular diseases. At least 50% of the population is below the age of 25 and the high prevalence of risk factors signal a massive onslaught of cardiovascular diseases in the next 10-15 years. As obesity, unhealthy diet, and high blood pressure have caused increases in the burden of CVD in the Middle East and North Africa (Zubaid et al., 2011). Cardiovascular diseases cause 45% of early deaths in the Gulf region. Around 30% of men and 44% of women in Saudi Arabia are obese and one-quarter of adults have diabetes (Zubaid et al., 2011, Alhyas et al., 2011). Although CVD and its risk factors are among the leading cause of death in many Middle East and East Mediterranean countries, very little is known about inter-ethnic differences in prevalence of risk factors, treatment response and survival. Ethno-cultural differences are known to be associated with cultural practices as well with lifestyles changes that might be related to different patterns of morbidity and mortality. The regions are home to various ethnic groups such as Arabs, Persians, Bedowins South Asian and European.

The limitations of our study must be considered. The number of subjects studied is small and this means they may not be representative of the general population. Also this limited statistical power to detect significant differences between groups, although using Cohen's d effect sizes helps to provide a handle on whether non-significant differences (due to lack of power) are likely to be physiologically relevant. In addition, it will be useful if we were able to measure post heparin LPL to detect any ethnic differences among the two groups.

Despite these limitations, few studies have documented a difference in the glycaemic response, and this might help to explain the high prevalence of diabetes in the Middle East and North Africa (Whiting et al., 2011).

In summary, results of the present study indicate that the more favourable lipoprotein profile found in over weight individuals Middle Eastern men and a trend of higher insulin response. Further studies are needed to explore these ethnic differences in more detail.

7 General Discussion

7.1 Summary

CVD is a major cause of death (WHO, 2014a). Alterations in lipid metabolism underlie atherosclerotic cardiovascular disease (Stegemann et al., 2014). Plasma TG concentrations consider as an independent risk factor in fasted (Hokanson and Austin, 1996) and postprandial state (Stampfer et al., 1996, Karpe, 1999). Hypertriglyceridemia is a prevalent risk factor for CVD and increasingly important in the setting of current obesity and insulin resistance epidemics. Recent data add confidence to the conclusion that TG levels appear to provide unique information as a biomarker of CVD risk because of their association with atherogenic lipoproteins and apolipoproteins, especially apo C-III (Luo and Peng, 2016). Accumulating evidence suggests that non-HDL-C, which combines the cholesterol levels found in LDL-C and TG-rich lipoproteins are an independent risk factor for atherosclerosis. Very low-density lipoprotein is the most atherogenic TRL particles, and the measurement of VLDL-C is relatively straightforward and captures distinct aspects of the TRL and plasma TG (Jialal and Devaraj, 2002). A large cross-sectional study (Penn Diabetes Heart Study (PDHS)) found that higher VLDL-C levels were associated with increasing coronary artery calcification after adjusting for numerous traditional CV risk factors in patients with type 2 diabetes (Prenner et al., 2014). Elevated TRL levels are associated with increased risks of CVD. VLDLs are the major component and atherogenic particles of TRL in the circulation (Takeichi et al., 1999, Kugiyama et al., 1999). A PDHS study that examined VLDL-C as an alternative marker of TRL demonstrated that VLDL-C levels are positively associated with increased coronary artery calcification in patients with type 2 diabetes even after adjusting for traditional risk factors (Prenner et al., 2014). Also, it has been reported that higher VLDL-C levels are associated with an increased risk of CVD in diabetic patients (Laakso et al., 1994). Several mechanisms have been proposed to explain why VLDL may be causally related to progression of atherosclerosis and CVD. VLDL is synthesized in the liver and serves as a vehicle that is responsible for the redistribution of triglycerides from liver to the peripheral tissues. Each VLDL particle contains a single molecule of apolipoprotein B100 and has a hydrophobic core that consists primarily of triglycerides and a small amount of cholesterol esters (Niu and Evans, 2011,

McEneny et al., 2000). The sizes of the VLDL particles vary depending on the quantity of triglycerides carried in the particle. Large VLDL particles are secreted from patients with hypertriglyceridemia and may contain 5 to 20 times more cholesterol than LDL. Unlike LDL, VLDL remnants rapidly penetrate the arterial wall, increase endothelial inflammation and facilitate the infiltration of monocytes, which results in foam cell formation and atherosclerosis (Nakamura et al., 2005, Nakajima et al., 2006). Hepatic VLDL overproduction has been reported to be related to insulin resistance (Sparks et al., 2012). It has been observed that in patients with metabolic syndrome or type 2 diabetes, insulin-resistant states and the loss of insulin-mediated suppression of apoB100 secretion lead to increased production of large VLDL₁ particles (Gill et al., 2004b, Adiels et al., 2006). Hypersecretion of large VLDL₁ particles, which contain more cholesterol, results in higher levels of remnant particles and small dense LDLs and lower levels of HDL that may contribute to the progression of vascular endothelial injury and atherosclerosis (Chen et al., 2012, Gianturco and Bradley, 1999).

To avoid the atherogenic cascade, TRL remnants must be cleared from plasma and metabolized by the liver before endothelial accumulation occurs. However, hypertriglyceridaemic states are associated with increased VLDL production and delayed VLDL clearance from circulation (Ooi et al., 2008, Zheng et al., 2010). In addition, delayed clearance of all apo-B lipoproteins, including VLDL, has been attributed to different mechanisms such as apo C-III-mediated inhibition of apoE and apoB-100 binding to hepatic receptors and proteoglycan; reduced activity of LPL, hepatic TG lipase; and finally, reduced apoE and apo E to C-III ratio reflecting reduced ability of the lipoproteins to be taken up by the liver. Similarly, CMs are taken up by the liver via LDL receptors or LDL receptor-related proteins and unless their TG core is removed they are not considered atherogenic due to their very large size, which slows entry into the arterial intima. It is the resulting CM remnants that are atherogenic, presumably because they are sufficiently small to pass through the endothelial cell barrier to the arterial intima (Gianturco et al., 1998). Hepatic lipase also plays a role in remnant removal (Cooper, 1997b) and its deficiency is associated with reduced TRL remnant clearance.

Lowering TRL has clearly been associated with benefit (Panel, 2002). Aside from lifestyle changes, further treatment with lipid-lowering drugs, such as statins, fibrates, nicotinic acid, or omega-3 fatty acids, may be warranted and should be considered to help achieve TRL targets in patients with hypertriglyceridemia. The

efficacies of pharmacologic interventions on TG level reduction vary depending on the agent used and the pre-treatment TG levels. Most cardiovascular diseases risks can be prevented by addressing behavioural risk factors such as tobacco use, unhealthy diet and obesity, physical inactivity. **Table 7.1.** shows the influence of behavioural changes on plasma TG. Treating obesity which is always linked to hypertriglyceridemia, could led to around 20% reductions on plasma TG, this reduction would be grater if individuals become physically active.

Diet modification also lowers plasma TG by 10-15%. The quality of fatty acids enhances plasma TG to a greater extends (Talayero and Sacks, 2011).

Table 7.1; Effects of lifestyle practices and lipid-lowering therapies on TG level reduction (Talayero and Sacks, 2011).

Therapeutic intervention	% TG reduction
Behavioural Risks	
Weight loss (5% to 10% total body weight)	20%
Diet	10-15%
Aerobic exercise (dependent on baseline TG levels)	15-20%
TG level > 1.69 mmol.l ⁻¹	
Coupled with weight loss	20-30%
Omega-3 PUFAs (dose dependent)	
Per every 1-g PUFA	5-10%
High-dose PUFA (3-4 g/d)	25-30%

In over this thesis we examined the effect of two behavioural change; diet and exercise and ethnicity on postprandial metabolism.

Studies observed a reduction of TG concentration prior exercise by ~ 20-25% (Gill, 2004, Gill et al., 2003, Malkova and Gill, 2006) and this reduction seems due to the increased clearance from the circulation of VLDL₁ (Al-Shayji et al., 2012).

The exact mechanism behind the increase of the clearance rate of VLDL₁ was not clear.

Thus, after developing a method to measure the affinity of TRL for LPL-mediated hydrolysis in **Chapter 3**, in **Chapter 4** the effects exercise on the affinity of TRL for LPL-mediated hydrolysis was assessed in 10 overweight/obese men. The results indicated that 90 minutes of prior moderate exercise increased the affinity of VLDL₁ for TG hydrolysis by lipoprotein lipase by 2.2-fold in the fasted state ($p = 0.02$) and 2.6-fold in the postprandial state ($p = 0.001$), but did not significantly

alter the affinity of chylomicrons or VLDL₂, this give an insight of a possible mechanism by which exercise can lower TG concentrations, and helps explain the observation that exercise appears to have a larger effect in lowering TG concentrations in VLDL₁ than in chylomicrons and VLDL₂ (Gill et al., 2006).

Co-ingestion of fat and carbohydrate reduced the postprandial glucose response significantly by 20%, but not the insulin. Also, it has been observed a substantial initial suppression of NEFA in the glucose trial with was markedly attenuated when glucose and fat were co-ingested. These changes might be due to genetic differences as the participants in all the studies were from different background. Also, we found the co-ingestions of carbohydrate with fat significantly decrease on VLDL1 by 13% ($p = 0.05$). This can explain some of the possible mechanism behind the long-term changes happened from the ingestion of different diet component.

In **Chapter 6** the metabolic responses to consumption of a mixed meal was assessed in eight white European men and eight men of Middle-Eastern origin. Postprandial insulin responses were higher in the Middle-Eastern men but, in contrast, postprandial TG concentrations were higher in the European men. Thus, the Middle Eastern metabolic response pattern is similar to the metabolic response of Black ethnic groups (Osei and Schuster, 1994). This knowledge can potentially help inform future strategies to mitigate metabolic disease risk in Middle Eastern populations by potentially considering interventions which have been effective in populations of Black ethnic origin and further research is needed to establish whether Middle Eastern groups share other metabolic risk characteristics with Black populations.

In all studies, Cohen's d effect sizes were used to provide additional insight into the likely physiological relevance of any differences observed which provides insights beyond whether an observation differs significantly between conditions (Cohen, 2013). While a P value can inform about the probability of a difference of the magnitude observed occurring in the absence of a true difference in the underlying population, it provides no information about the size of the effect. With a large enough study clinically trivial differences could be statistically significant. Conversely, for variables with large variability, it is possible to miss potentially clinically relevant findings, which may have been statistically

significant with a larger sample size, by simply considering statistical significance at the conventional threshold of $p < 0.05$. This was particularly relevant in **Chapter 5**, where some variables did not differ significantly between the two ethnic groups, but large effect sizes were seen.

7.2 Limitation of the studies

As with any scientific study there were limitations to the research undertaken in this thesis and it is important to acknowledge these. One of the limitations of the method we used to measure the affinity of TRL for LPL (**Chapter 4**) is the large amount of blood we were using which was an obstruction to take postprandial blood samples beyond 4 hours. It is possible that some individuals experienced peak TG concentrations after 4 hours, so this would have been missed in the experimental design. However, concentrations of TG were substantially higher at 4 hours postprandially compared with the fasted state and chylomicron concentrations were high at this point. Importantly, the effects of exercise on the affinity of VLDL₁ for LPL-mediated TG hydrolysis were similar in the fasted and postprandial states, so it does seem likely that this may not have been a major concern. The trial was time consuming; sample processing and analysis required a full week of laboratory work after each study day with participants. This, and other logistic factors, limited the scope to undertake further measurements which may have helped to provide an even more complete picture of the effects of exercise on postprandial lipoprotein metabolism. For logistic reasons including the large amount of blood was withdrawn it was not possible to undertake a kinetic study in parallel to also determine the rate of production and clearance of TRL species. This would have enabled correlation of the extent to which change in affinity of lipoprotein particles for LPL-mediated TG hydrolysis paralleled any differences in rates of lipoprotein clearance. It was also not possible to measure the post-heparin LPL activity for two main reasons. The first was the need for clinical cover during heparin injections which was not available for these studies. The second was that injection of heparin disturbs lipoprotein metabolism, thus to obtain a picture of the effects of exercise on LPL activity in the fasted as well as postprandial states, it would have been necessary for each participant to undertake the overall protocol twice - once with fasting measurements of LPL activity made in the presence and absence to exercise and a second time with LPL activity measurements made in the postprandial state. It was felt that this would

have induced too much burden of participants who were already undertaking a challenging research protocol. Again, this limited the potential to understand the extent to which any difference in TG clearance may have been mediated by altered lipoprotein affinity for LPL vs an increase in LPL activity. We also did not evaluate the effects of exercise on meals with different compositions of fatty acids which could conceivably influence the affinity of chylomicrons for LPL-mediated TG hydrolysis.

For the experiment in **Chapter 5**, the aim was to determine in principle the effects of fat and carbohydrate consumed separately or together on postprandial metabolism. To maximise the potential effects the meals used were extreme ones, with a very large fat intake (75g) and the carbohydrate consumed being all glucose. Thus does not fully reflect the actual meals that are consumed in the daily life. Thus the postprandial responses to meal ingestion in real life setting are likely to be less pronounced than the ones observed here. In addition, only one composition of fatty acids (high in SFA) was considered, and responses to different fatty acid compositions may be different. In terms of sample analysis, we didn't measure apoB concentrations in lipoprotein particles and total, HDL and sdLDL in postprandial state.

A key limitation in the study in **Chapter 6** was that the small sample size may have limited our ability to detect more robust associations between ethnicity and differences in postprandial response. Larger studies are needed to confirm the findings that were observed. In addition, we did not ask the participant to follow unified controlled diet during the three days prior the trial, so differences between the two ethnic groups in habitual diet may have influenced the findings. However, there is no simple solution here as providing all participants with the same preceding diet may reflect a dietary intervention if this diet differs substantially from what participants usually eat. Some might argue the differences between the two groups in the baseline measurements lead to the different postprandial responses between the two groups. However, any difference in fasting measure would also reflect an ethnic difference and attempting to control for this may not be helpful. Participants were matched for age and BMI, thus also attempting to match for baseline metabolic characteristics may result in participants who are atypical for their given ethnicity. This has been shown in many studies comparing ethnic groups, measuring factors such as mineral ion

handling (Gutiérrez et al., 2010), glycaemic response (Venn et al., 2010, Likhari and Gama, 2009) and lipaemic response (Punyadeera et al., 2001, Sharrett et al., 2001). For the postprandial response one of the factors might led to different response between the two groups is that two different meals consumed (however, there was no significant statistical differences between the different meals for any of the responses measured).

For all studies, we hoped to measure many different metabolites using a nuclear magnetic resonance (NMR) platform - a novel technology which enables simultaneous detections of over 200 metabolites within a single small amount of blood (Ala-Korpela et al., 2012). This technique would have yielded further insights into the effects of exercise, dietary intake and ethnicity on postprandial responses, but unfortunately, these methods were not up and running in Glasgow within the timeframe needed for inclusion in this thesis.

7.3 Future experiments

Taken together, these studies provide new insights in the role of dietary intake, exercise and ethnicity on postprandial responses. This will increase our understanding in the changes that occur in the postprandial state. Going forward there are a number of factors we can consider with respect to study of exercise and lipoprotein metabolism; one factor that can be taken into consideration is evaluating the competition of the affinity between VLDL₁ and chylomicron by incubating both particles *in vivo* assay. It is well established that chylomicron affinity for LPL-mediated hydrolysis is greater than VLDL₁ affinity, which is in turn greater than VLDL₂ affinity (Fisher et al., 1995). This can done by radiolabelling VLDL₁ particles taken from exercise and non-exercised conditions co-incubating with chylomicrons and assessing their affinity for LPL-mediated TG hydrolysis under this competitive situation which more closely reflects *in vivo* physiology. Also, it would be helpful to perform a kinetic study in which VLDL is subdivided into more than the two standard subfractions to provide a clearer insight into how exercise may be influencing affinity of VLDL₁ for clearance - for example by altering the size distribution of particles within the VLDL₁ range. Although an increase of LPL activity does not always accompany the exercise-induced reductions in postprandial TG (Malkova et al., 1999, Herd et al., 2001, Miyashita and Tokuyama, 2008), past literature did report such changes in some cases while

not in other cases (Herd et al., 2001), it would be helpful to measure lipoprotein lipase activity in this same study to reach to a final conclusion. Also, investigating the lipaemic response to differing types of exercise in individuals who have or are at risk for developing metabolic disease, including female participants and individuals who are under the age of 18 years or over the age of 40 years, will broaden the understanding of exercise and the postprandial lipaemic response.

It would be helpful to examine the effects of ingesting different ratios of fat and carbohydrate, and different qualities of fat and carbohydrate, with respect to fatty acid composition and glycaemic index on postprandial metabolic responses. Here examining NMR metabolomic responses will provide a step-change in the ability to characterise the metabolic responses to fat and carbohydrate ingestion and the time-based nature of postprandial interactions between metabolites. Future research should aim to gain further understanding of exercise-nutrition interactions, such as peri-exercise carbohydrate availability which may help to refine interventions and future public health guidelines.

Future research should focus on large enough samples to be able to evaluate genetic-lifestyle effects (Crook and Taylor, 2003), although this may be difficult to do with large enough samples, given the labour-intensive nature of these investigations.

Comparison studies similar ethnic groups in different environments provide powerful epidemiological tools and thus further study could include studying individuals of Middle Eastern descent living in the Middle East and in the UK.

More detailed measurements of lipoprotein metabolism as described above (i.e. kinetic studies, LPL measurement, NMR metabolomic measurements) will help provide further insights into differences between the Middle Eastern and European men, and of course, it is important to replicate these studies in women. Moreover, it will be interesting to determine the effect of exercise and dietary interventions on metabolic responses among this population. Given what has been observed on experimental **Chapter 6**, it would be great to match the participants for their fasting TG level and investigate the influence of the ingestion of high fat meal on postprandial TG responses and TG-rich lipoprotein metabolism.

7.4 Conclusion

In conclusion, this thesis has provided novel insights into understanding to lipid and carbohydrate metabolism in response to different factors. The first study showed that the affinity of VLDL₁ increased to LPL enzyme post-exercise, which may explain, at least in part, the mechanism by which exercise reduces plasma TG. In addition fasting and postprandial sdLDL concentrating was significantly lowered post-exercise. The second study aimed to investigate the metabolic responses to three different meal compositions (fat only, carbohydrate only and combination of equal amount of both). Although, the plasma TG concentrations were unexpectedly similar in fat and combination meal, the glycaemic response was significantly lower when fat and carbohydrate were ingested together. Finally the third study was a pilot study which investigated the ethnic differences between recruited Middle Eastern and recruited European in their metabolic response to a mixed meal. This study revealed potential differences in TG and glucose metabolism between the two ethnic groups, where fasting and postprandial TG were significantly higher European (almost double) than that of Middle Eastern. Although, there was no significant different in fasting glucose concentrations between the two ethnic groups, the postprandial response tended to be slightly higher in the Middle Eastern group. Interestingly, the insulin concentration was 3 and 4 times higher in fasted and postprandial states in Middle Eastern compared to European, although this it did not reach statistical significance, likely due to the relatively small sample size. Thus, taken together, the results of this thesis add to the basic science understanding of the effect of exercise, dietary intake and ethnicity on postprandial metabolism.

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9 Appendices

Appendix A; Heath Screen Questionnaire -Chapter 4, 5 & 6



University of Glasgow | College of Medical,
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HEALTH SCREEN FOR STUDY VOLUNTEERS

Name: _____

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate:

1. **At present**, do you have any health problem for which you are:

- | | | |
|--|---------|--------|
| (a) on medication, prescribed or otherwise | yes [] | no [] |
| (b) attending your general practitioner | yes [] | no [] |
| (c) on a hospital waiting list | yes [] | no [] |

2. **In the past two years**, have you had any illness which required you to:

- | | | |
|---|---------|--------|
| (a) consult your GP | yes [] | no [] |
| (b) attend a hospital outpatient department | yes [] | no [] |
| (c) be admitted to hospital | yes [] | no [] |

3. **Have you ever** had any of the following:

- | | | |
|--|---------|--------|
| (a) Convulsions/epilepsy | yes [] | no [] |
| (b) Asthma | yes [] | no [] |
| (c) Diabetes | yes [] | no [] |
| (d) A blood disorder | yes [] | no [] |
| (e) Digestive problems | yes [] | no [] |
| (f) Disturbance of balance/co-ordination | yes [] | no [] |
| (g) Numbness in hands or feet | yes [] | no [] |
| (h) Disturbance of vision | yes [] | no [] |
| (i) Thyroid problems | yes [] | no [] |
| (j) Kidney or liver problems | yes [] | no [] |

- (k) Chest pain or heart problems yes [] no []
- (l) Any other health problems yes [] no []

4. Have any of your family (parents, grandparents, brothers, sisters, children, aunts, uncles, cousins) ever had any of the following: (if yes please give details including age of first diagnosis)

- | | | |
|--------------------------------|------------|-----------|
| (a) Any heart problems | yes [] | no [] |
| (b) Diabetes | yes [] | no [] |
| (c) Stroke | yes [] | no [] |
| (d) Any other family illnesses | yes [] | no [] |

5. Do you currently smoke yes [] no []
Have you ever smoked yes [] no []

If so, for how long did you smoke and when did you stop?

6. How many units of alcohol do you typically drink in a week?
7. Have you taken part in a research study in the last 3 months? yes [☐]
no [☐]

If YES to any question, please describe briefly if you wish (e.g. to confirm whether problem was short-lived, insignificant or well controlled.) (Use a separate sheet if necessary)

.....

.....

Blood pressure measured at screening.....mm Hg

Blood Glucose measured at screening.....mmol.l⁻¹

Appendix B1; Subjects Information Sheet and consent Form -Chapter 4



University of Glasgow | College of Medical,
Veterinary & Life Sciences

VOLUNTEER INFORMATION SHEET

Title: A Pilot Study to Determine the Effect of Exercise on the Affinity of Lipoprotein for lipoprotein Lipase

Lay title: Effect of Exercise on Fats in the Blood

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of the study?

Heart disease is the leading cause of death in Scotland. A high level of fat in the blood is an important risk factor. We know exercise reduces the level of fat in the blood, but we don't know fully understand how it does this. Recent research from our lab suggested that mild exercise changes the nature of fat particles in the blood stream so that they are more easily cleared away. This study will help to increase understanding of how this happens. This is important as exercise could be used as a treatment option, rather than drugs, to prevent and treat disturbances in blood fat metabolism.

Why have I been chosen?

You have been chosen because you are a healthy man aged between 18-60 years, and you are heavier than the ideal weight for your height.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide, you will be given this information sheet to keep and be asked to sign a consent form. If you do this you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

In the first instance you will be asked to attend for a screening visit in which we will:

- discuss with you and complete confidential questionnaires regarding your health, family history and physical activity level
- measure your blood pressure
- take your height, weight and waist measurements
- take a small blood samples to check the fat and sugar levels in your blood.
- provide an opportunity for you to ask questions

These preliminary procedures will enable us to determine whether you fall into the group of people we wish to study and will also ensure that it is perfectly safe for you to take part.

Experimental procedures

A. Preliminary Exercise Test

At the beginning of the study, an exercise test will be undertaken. This will involve walking on a motorised treadmill. If you are not used to walking on a treadmill, we will familiarise you with this before any 'real' sessions are performed. The test is designed to estimate your body's ability to use oxygen and enables us to find the correct speed and gradient for you to walk at during your treadmill walks. This will not require a maximal effort and the test will last for about 20 minutes. Heart rate will be monitored and recorded throughout using a heart rate monitor and expired air will be collected at intervals using a mouthpiece and respiratory valve. For safety reasons, the test will be stopped if your heart rate exceeds 85% of your predicted maximum.

B. Body Composition

The amount and distribution of your body fat will be determined by measuring body girths and by using callipers to measure skin fold thickness at four different sites (a sophisticated version of "pinch an inch"). Your height, weight and waist and hip circumferences will also be recorded. You will need to wear only underclothing for these measurements which will be made in private by an experienced researcher. These measurements only take a few minutes and can be made on the same day as other tests.

C. Main Trials

We will ask you to undertake two trials in random order. The first trial is a control with no exercise and the other is by performing a single exercise session. On the day prior the test, a controlled exercise session of 90-minute walk will be undertaken (Exercise test). Other than this, conditions (such as alcohol consumption, food eaten, etc) in the days leading up to each trial will be EXACTLY the same. On day 2 you will report to the lab after ~12 h fasting, a cannula will be inserted into an antecubital or forearm vein. No more than 120 ml (approximately 8 tablespoons) of blood will be taken in total during the course of this trial (less than quarter the volume of blood given in a blood donation) and a fatty meal will be provided as an oral fat tolerance test (OFTT). (Please refer to **what do I have to do** section).

Control Trial (con): on day one you will be asked to stay at home without doing any exercise. On day 2 you will come to the Clinical Investigation Suite (or whatever it's called) after approximately 12hours fasting overnight. After giving a blood sample we will provide you with a creamy milkshake and buttered croissant, we will have 4 more blood samples at 30 min, 0hr, 2hr and 4hr. You will

remain fasting but we will provide you with plenty of water or low you are free to sit, read, relax, or watch TV during the entire observation.

Exercise trial:

This trial will be identical to the control trial except on the Day 1 you will visit the exercise laboratory in the evening and complete a treadmill walk at brisk force representing half of your maximum capacity.

What do I have to do?

We also ask you to maintain your usual lifestyle (i.e. don't change your diet or exercise habits) for the duration of this study. However, before each trial, we ask you to do the following:

1. For 3 days before each trial, refrain from planned or strenuous exercise, other than for personal transportation and the 90-minute treadmill walk in our laboratory.
2. Weigh and record everything you eat and drink for 2 days before each test. We will provide you with weighing scales and diet sheets to do this.
3. Refrain from alcohol consumption on the day before each test.
4. For the 2 days leading up to the second test, we will ask you to eat the same diet as you did on the two days leading up to the first test. Therefore, we would advise you to eat meals that you will be able to easily repeat during the days preceding both tests.
5. FASTING

What are the possible disadvantages and risks of taking part?

- Exercise testing will not be at a maximal level but the possibility exists that, very occasionally, certain changes may occur during or shortly after the tests. They include abnormal blood pressure, fainting or a change in the normal rhythm of the heartbeat. Reassure them that they will be looked after
- Blood sampling via the cannula may cause minor bruising or an inflammation of the vein. Good practice, however, minimises this risk. Some people may feel faint when they give blood.

What are the possible benefits of taking part?

There may be no immediate benefits to you personally, but as a result of being involved in this study you will receive health and fitness information about yourself including fitness tests, dietary assessment, body fat measurement and your cholesterol and blood sugar levels. This study will help us to determine how exercise can improve risk factors for heart disease and diabetes. The findings will be published in scientific journals so that understanding of the way in which exercise decreases the risk of heart disease and diabetes can be increased. This information may help make up better exercise guidelines, particularly for people who are overweight or obese.

We will provide you with feedback about the main study findings and also about your own results and would be delighted to explain results and discuss the implications with you.

What if something goes wrong?

The chances of something going wrong are extremely small. We have recently conducted a similar project and there were no problems. All of the procedures involved in this study are low risk and our screening tests are designed to ensure that you will only participate if it is safe for you to do so. In the unlikely event

that you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it.
 Reassurance again re experienced researchers

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the University or hospital will have your name and address removed so that you cannot be recognised from it. In addition, your records, samples and results will be identified by a number and not your name. where will it be stored?

What will happen to the results of the research study?

The results from this study will be presented at scientific meetings and published in scientific journals. A copy of the published results will be sent to you upon request. You will be informed which part of the study you were in, as this information will be confidential and no one else will know your name and which part you participated in. thesis?

What will happen to my samples after the study has finished?

The blood samples that you provide for this study may be useful for future research into the prevention and treatment of diabetes and heart disease; this may involve investigating new biochemical markers that are not yet identified. Samples will be analysed anonymously and will require a new ethics application before they would be used for future research. If you do not wish your samples to be used for future research, please indicate this on the consent form.

Who has reviewed the study?

College of Medical veterinary and Life Science (MVLS), ethics committee.

Contact for Further Information

Any questions about the procedures used in this study are encouraged. If you have any doubts or questions, please ask for further explanations by contacting either:

Khlood Ghafouri
 Tel: 07868351398

E-mail: k.ghafouri.1@research.gla.ac.uk

Dr Jason Gill

Tel: 0141 3302916

E-mail: Jason.Gill@glasgow.ac.uk

You will be given a copy of this information sheet and a signed consent form to keep for your records.



Volunteer Identification Number for this trial: _____

CONSENT FORM

Title of Project: A Pilot Study to Determine the Effect of Exercise on the Affinity of Lipoprotein for lipoprotein Lipase

Lay title: Effect of Exercise on Fats in the Blood

Name of Researcher: _____

Please initial box

1. I confirm that I have read and understand the information sheet datedfor the above study and have had the opportunity to ask questions.

☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

☐

3. I agree to take part in the above study.

☐

4. I agree for my samples to be used for future research into the prevention and treatment of diabetes and heart disease. This may involve analysis of new biochemical markers not yet identified.

Yes

☐

No

☐

Name of Subject

Date

Signature

Name of Person taking consent
(if different from researcher)

Date

Signature

Researcher

Date

Signature

Copy for subject
Copy for researcher

Appendix B2; Subjects Information Sheet and consent Form -Chapter 4



University of Glasgow | College of Medical,
Veterinary & Life Sciences

VOLUNTEER INFORMATION SHEET

Title: A pilot study to determine metabolomic responses to fat and carbohydrate ingestion

Lay title: Metabolic responses to consuming fat and sugar

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of the study?

Heart disease and diabetes are leading causes of death and ill health in Scotland. High levels of fat and sugar in the blood, particularly after we eat food, are important risk factors for these conditions. Many different factors in the blood change when we eat, but until recently it was difficult to measure all of them. With new technology it has become possible to measure dozens of different factors in the blood related to fat and sugar metabolism in a single blood sample. Measuring how all of these factors change after eating different types of meals help our understanding of how fat and sugar consumption can influence risk for heart disease and diabetes.

Why have I been chosen?

You have been chosen because you are a healthy adult aged between 18-60 years.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to participate, you will be given this information sheet to keep and be asked to sign a consent form. If you do this you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

In the first instance you will be asked to attend for a screening visit in which we will:

- discuss with you and complete confidential questionnaires regarding your health, family history and physical activity level
- measure your blood pressure
- take your height, weight and waist measurements
- take a small blood samples to check the sugar level in your blood.

- provide an opportunity for you to ask questions

These preliminary procedures will enable us to determine whether you fall into the group of people we wish to study and will also ensure that it is perfectly safe for you to take part.

Experimental procedures

We will ask you to undertake three experimental trials in random order, separated by an interval of 1-3 weeks, as described below.

Glucose test: We will ask you to come to the University after an overnight fast (i.e. having eaten nothing for 12 hours) and spend about 9 hours with us in our metabolic investigation suite. We will give you a sugary drink containing 75 g (15 teaspoons) of glucose and will take small blood samples before the meal and at intervals for 8 hours afterwards to determine how your body is handling the food. Taking blood will be no more painful than a simple blood test as samples will be obtained from a tiny plastic tube called a ‘cannula’ placed in a forearm vein. A total of 140 ml (about 9 tablespoons, or less than a third of the amount taken when you donate a “pint” of blood) will be taken over the course of the day. During your time with us, you will be able to rest comfortably in our metabolic investigation suite, watching TV or videos, reading or working. At the end of the experiment we will give you a meal to eat before you go home. On the morning of the next day we will ask you to come back to the lab after an overnight fast for one further blood sample.

Fat test: This will be identical to the glucose trial except we will give you a creamy milkshake to drink that contains 75 g of fat.

Combined fat and glucose test: This trial will be identical to the previous trials except we will give you both drinks together.

What do I have to do?

Other than the specific tasks described above, we ask you to maintain your usual lifestyle (i.e. don’t change your diet or exercise habits) for the duration of this study. We will ask you to weigh and record everything you eat and drink for two days before your first main trial, and not to drink alcohol or exercise on these days, and to repeat this diet before your second and third main trials. We will provide you with weighing scales and diet sheets to do this.

What are the possible disadvantages and risks of taking part?

- Blood sampling via the cannula may cause minor bruising or an inflammation of the vein. Good practice, however, minimises this risk. Some people may feel faint when they give blood.
- There is a small possibility that taking part in this study will reveal a health problem that you already have such as high cholesterol or high blood pressure. If such a problem is revealed, we will ask your permission to inform your GP to ensure that you receive appropriate treatment.

What are the possible benefits of taking part?

There may be no immediate benefits to you personally, but as a result of being involved in this study you will receive health information about yourself including dietary assessment and your cholesterol and blood sugar levels. This study will help us to determine how fat and sugar intake influences risk factors for heart disease. The findings will be published in scientific journals so that understanding of the way in which diet influences the risk of heart disease and diabetes can be increased.

We will provide you with feedback about the main study findings and also about your own results and would be delighted to explain results and discuss the implications with you.

What if something goes wrong?

The chances of something going wrong are extremely small. We have conducted several similar projects over the past 10-15 years, with many hundreds of participants, and have never had any problems. All of the procedures involved in this study are low risk and our screening tests are designed to ensure that you will only participate if it is safe for you to do so. In the unlikely event that you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the University or hospital will have your name and address removed so that you cannot be recognised from it. In addition, your records, samples and results will be identified by a number and not your name.

What will happen to the results of the research study?

The results from this study will be presented at scientific meetings and published in scientific journals. The results will also form part of Miss Khlood Ghafouri's PhD thesis. A copy of the published results will be sent to you upon request. You will not be identifiable in any of the data presented or published from this study.

What will happen to my samples after the study has finished?

The blood samples that you provide for this study may be useful for future research into the prevention and treatment of diabetes and heart disease; this may involve investigating new biochemical markers that are not yet identified. Samples will be analysed anonymously and will require a new ethics application before they would be used for future research. If you do not wish your samples to be used for future research, please indicate this on the consent form.

Who has reviewed the study?

This study has been reviewed and approved by the College of Medical Veterinary and Life Sciences Ethics Committee at the University of Glasgow.

Contact for Further Information

Any questions about the procedures used in this study are encouraged. If you have any doubts or questions, please ask for further explanations by contacting either:

Khlood Ghafouri

E-mail: k.ghafouri.1@research.gla.ac.uk

Tel: 0141 2329494 (office) or 07868351398 (mobile)

Dr Jason Gill

Tel: 0141 3302916

E-mail: jason.gill@glasgow.ac.uk

You will be given a copy of this information sheet and a signed consent form to keep for your records.



Volunteer Identification Number for this trial: _____

CONSENT FORM

Title: A pilot study to determine metabolomic responses to fat and carbohydrate ingestion

Lay title: Metabolic responses to consuming fat and sugar

Name of Researcher: _____

Please initial box

1. I confirm that I have read and understand the information sheet dated

for the above study and have had the opportunity to ask questions.

☐

5. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

☐

6. I agree to take part in the above study.

☐

7. I agree for my samples to be used for future research into the prevention and treatment of diabetes and heart disease. This may involve analysis of new biochemical markers not yet identified.

Yes

☐

No

☐

Name of Subject

Date

Signature

Name of Person taking consent
(if different from researcher)

Date

Signature

Researcher

Date

Signature

Copy for subject
Copy for researcher

Appendix C; Blood and lipoprotein subfraction analysis

Spectrophotometric Assays

Plasma analyses were carried out using commercially available kits. Plasma glucose (Randox Laboratories Ltd, UK) and total and HDL cholesterol (Roche Diagnostics, UK,) were analysed in the fasted state. LDL cholesterol was calculated in the fasted state using the Friedewald equation (Friedewald et al., 1972). TG (Randox Laboratories, Crumlin, UK), NEFA (Wako Chemicals, GmbH, UK, LTD).

Samples were analysed in duplicate on an automated spectrophotometric IL600 Analyser (Instrumentation Laboratories, USA). The IL600 is an automated, random access clinical chemistry analyser which uses analytical technique photometry for the in vitro quantitation of analyses found in physiological fluids, such as serum, plasma, urine or cerebrospinal fluid.

All the samples were analysed in **McGregor Building, University of Glasgow** by Mrs. Josephine Cooney.

Method and materials

The following materials and equipment were used in all testes.

Equipment

- IL600 Analyser (Instrumentation Laboratories, USA).
- 2 ml Free standing Apex tubes (Alpha Laboratories).
- Hitachi sample cups (Sarstedt Ltd).

Samples

- 500 µl Plasma and serum samples were stored at 4 °C fridge and/or frozen at -80 °C.

Quality Control

- Each test QC were prepared and stored as 350 µl aliquots in Apex tubes at -80 °C.

Procedure

The following steps were applied in each test:

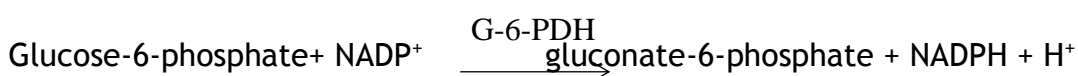
1. QC and standers for each test were prepared according to the insert inside each kit and stored as recommended.
2. Reagents were prepared freshly before each test.
3. QC, stander and samples were defrosted (or removed from the fridge) for 45 minutes, then mixed gently.
4. The IL600 analyser was calibrated before each test was started.
5. Samples and reagent were loaded into the sample tray as per worksheet. A minimum sample volume of 200 μ l is required and the test was started by sending an order via computer system.
6. The samples and QC were incubated in for 5 minutes at 37 °C and the absorbance were read at the required wave length.
7. The values of the samples and QC were determined by the analyser using a regression equation from analysis of the standers.
8. QC values were checked against acceptable ranges in the kit inserts in the stock book or by using the QC package on Modulab. If QCs were out of acceptable range, the IL600 was recalibrated and the samples were retested, if the QC was still out, a further sample aliquot was used and the reagents were checked for contamination.

Determination of plasma glucose concentration

Plasma glucose concentrations were determined in by enzymatic method using commercially available kits (Glucose hexokinase, Randox Laboratories, Crumlin, UK). Samples were analysed in IL600 as mentioned above.

Principle

The enzymatic hexokinase (HK) catalyses the reaction between glucose and adenosine triphosphate (ATP) to form glucose-6phosphate and adenosine diphosphate (ADP). In the presence of NAD, the enzyme glucose-6-phosphate dehydrogenase (G6PDH), oxidizes glucose-6-phosphate to 6-phosphogluconate. The increase in NADH concentration is directly proportional to the glucose concentration and can be measured photometrically using the IL600 analyser at 340 nm. The equation is shown below:



The CV for the assay was 2.0%.

Reagents

- Glucose Kit. (Randox Laboratories, Northern Ireland).
- R1- Buffer/Coenzymes (contents ready for use).
- R2 -made by mixing Reagent 2 diluent to one bottle of R2 enzymes rinsing the contents several times with the diluent.

Standards

- Randox Calibration Serum Level 2 was reconstituted in 3 ml of distilled water at room temperature with gentle mixing.

Quality Control

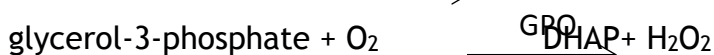
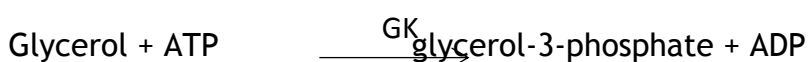
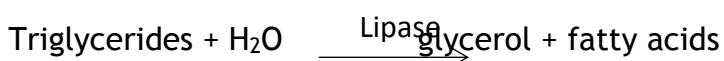
Human Assay Control (HC2 and HC3 Catalogue No. HS2611, Randox, Crumlin, UK), were prepared by mixing each control into 5 ml of distilled or deionised water.

Determination of plasma and lipoprotein triglyceride

Plasma and lipoprotein TG concentrations were determined by an enzymatic method using commercially available kits (GPO-PAP, Randox Laboratories, Crumlin, UK). Samples were analysed in IL600 as mentioned above.

Principle

The method based on lipase hydrolysis of TG to glycerol followed by oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide produced then reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase to form a red dyestuff which was measured spectrophotometrically using the IL600 analyser at 550 nm. The equation is shown below:



(GK: Glycerol kinase; GPO: Glycerol phosphate oxidase; POD: Peroxidase; DHAP: dihydroxyacetate phosphate). The CV for the assay was 3.8%.

Reagents

- Triglyceride Reagent (Randox laboratories Reagent Kit Cat No TR210ridge) was made by dissolving R1b on 15 ml of R1a.
- Sodium Chloride (NaCl) Cat No: S9888 Sigma.

Standards

- 0.9% NaCl (used as Saline Blank).
- Ready to use calibrator

Quality Control

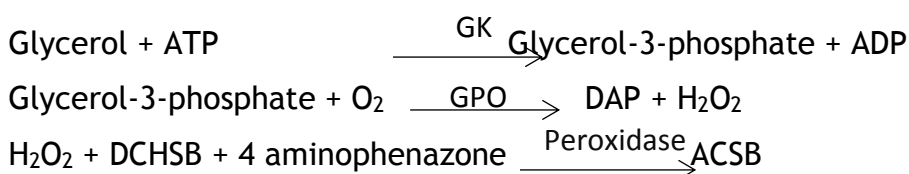
Two controls were included in the kit. Normal lipid control (LPD1) and abnormal Lipid control (LPD3)(Catalogue No LE 2661 and No LE 2663, Randox Laboratories, Crumlin, UK. Each control has been reconstituted in 3 ml distilled or deionised water.

Determination of plasma glycerol

Determinations for Glycerol were performed using commercially kits (Randox Laboratories, Crumlin, UK). Samples were analysed in IL600 as mentioned above.

Principle

A direct spectrophotometric procedure for the measurement of glycerol utilising a quinoneimine chromogen system in the presence of glycerol kinase, peroxide and glycerol phosphate oxidase. The equation is shown below:



(GK: Glycerol kinase; GPO: Glycerol phosphate oxidase; DCHBS: 3,5-dichloro-2-hydroxybenzene sulphonic acid; ACSB: n-(4-antipyril-3-chloro-5-sulphonate-p-benzoquinoneimine). The CV for the assay was 1.9%.

Reagents

Randox glycerol Colorimetric Assay (cat no GY105, Randox Laboratories, Crumlin, UK).

Reagent 1-Buffer as supplied

Reagent 2-4-aminophenazone color reagent lyophilised.

Glycerol reagent prepared by adding 15 ml of reagent 1 to one bottle of reagent 2, then mixed gently and use for analysis.

Standards

100 $\mu\text{mol.l}^{-1}$ standard supplied ready to use.

Quality Control

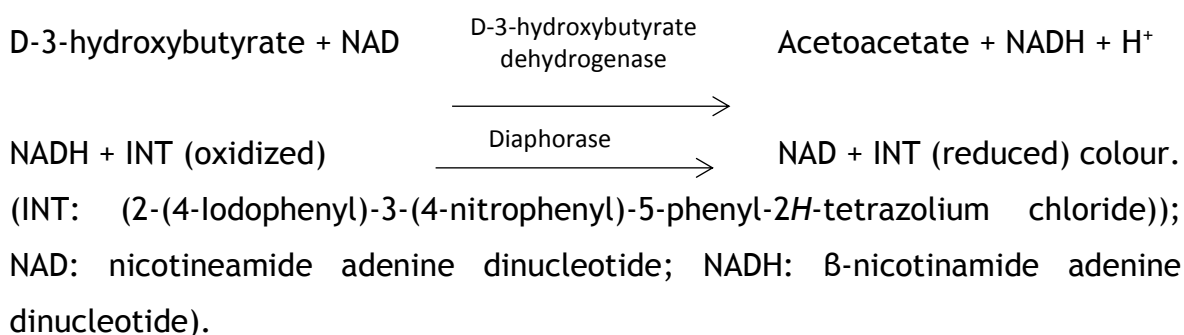
Randox glycerol (Control cat no GY1369, Randox Laboratories, Crumlin, UK).

Determination of plasma hydroxybutrate Analysis

Determinations for hydroxybutrate were performed using commercially available (Wako Chemicals, GmbH, UK, LTD). Samples were analysed in IL600 as mentioned above.

Principle

The principle of this test is based upon the enzymatic conversion of β -hydroxybutyrate dehydrogenase to acetoacetate by the action of D-3-hydroxybutyrate dehydrogenase, and concomitantly the co-factor nicotinamide adenine dinucleotide (NAD^+) is converted to its reduced form β -nicotinamide adenine dinucleotide (NADH). In the presence of diaphorase, NADH reacts with the colorimetric detector WST-1 to produce a formazan dye with a maximum absorbance at 505 nm. The equation is shown below:



Reagents

R1 containing β -hydroxybutyrate dehydrogenase and diaphorase enzymes.

R2 containing NAD, INT and oxalate.

Reagent prepared by mixing 10 ml of reagent 1 with 1.5 ml of reagent 2.

Standards

Standard containing 1mM sodium D-3-hydroxybutyrate was provided in the kit.

Quality Control

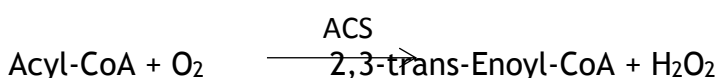
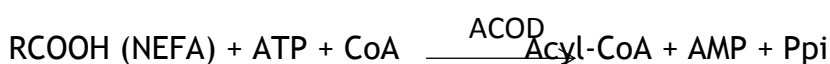
Radox glycerol (Control cat no GY1369, Radox Laboratories, Crumlin, UK).

Determination of plasma NEFA Analysis

Determinations for NEFA were performed using commercially available (Wako Chemicals, GmbH, UK, LTD). Samples were analysed in IL600 as mentioned above.

Principle

This method relies upon the acylation of coenzyme A (CoA) by the fatty acids in the presence of added acyl-CoA synthetase (ACS). The acyl-CoA thus produced is by added acyl-CoA oxidase (ACOD) with generation of hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase (POD) permits the oxidative condensation of 3-methyl-N-ethyl-N (B-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple colour adduct which can be measured spectrophotometrically at 550 nm. The equation is shown below:



(ACS: acyl-CoA synthetase; ACOD: acyl-CoA oxidase; MEHA: 3-methyl-N-ethyl-N-B-hydroxyethyl-aniline). The CV for the assay was 5.2%.

Reagents

Two colour reagents (R1A and R2A) were supplied in powder form with the kit. For analysis performed, the reagent R1A was reconstituted in 50 solvent R1. Colour reagent R2A, reconstitute with 20ml of solvent. Standard Solution is (1.0 mEq.L⁻¹)

Standards

Standard supplied with kit.

Quality Control

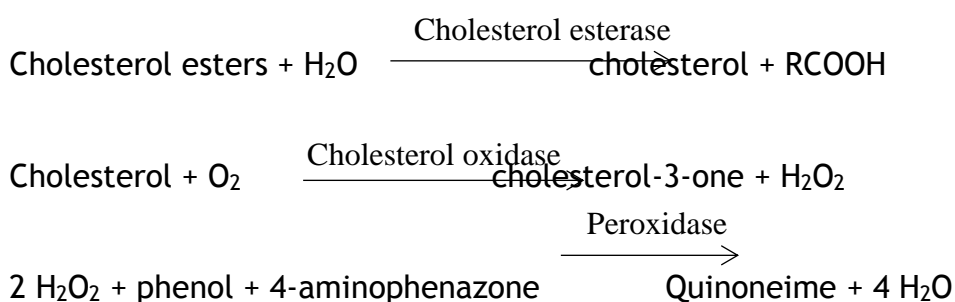
Human Assay Control (HC2 andd HC3 Catalogue No. HS2611, Randox, Crumlin, UK), were reconstituted by mixing each control into 5 ml of distilled or deionised water.

Determination of plasma and lipoprotein total cholesterol

Total plasma cholesterol was determined using commercially available kits (Randox Laboratories, Crumlin, UK). Samples were analysed in IL600 as mentioned above.

Principle

The principle based on the generation of hydrogen peroxide from the substrate by the action of cholesterol esterase, after the hydrolysis of EC, which is coupled through peroxidase to produce a chromogen, detected by its absorbance at 500 nm. The equation is shown below:



The CV for the assay was 2.9%.

Reagents

- Cholesterol Reagent (Reagent Kit Cat No CH200, Randox Laboratories, Crumlin, UK), used as supplied.
- Sodium Chloride (NaCl) Cat No: S9888 Sigma

Standards

- 0.9% NaCl (used as Standard Blank).
- Calibrator (CFAS, Cat. No.10759350, Roche).
- The calibrator was reconstituted in 3 ml of distilled water at room temperature with gentle mixing.

Quality Control

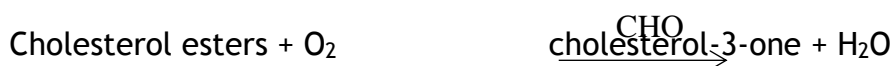
Two controls were included in the kit. Normal lipid control (LPD1) and abnormal Lipid control (LPD3)(Catalogue No LE 2661 and No LE 2663, Randox Laboratories, Crumlin, UK. Each control has been reconstituted in 3 ml distilled or deionised water.

Determination of plasma and lipoprotein free cholesterol

Plasma and lipoprotein free cholesterol concentrations were determined by an enzymatic method using commercially available kits (Diagnostic Systems GmbH, Germany). Samples were analysed in IL600 as mentioned above.

Principle

Free cholesterol in the serum is oxidised by cholesterol oxidase to cholestenone and produces simultaneously hydrogen peroxide. The hydrogen peroxide formed causes phenol and 4-aminoantipyrine to undergo quantitatively an oxidative condensation in the presence of peroxidase, to produce a red colour. The amount of free cholesterol in the test sample is determined by measurement of the absorbance of the red colour at 510 nm. The equation is shown below:



The CV for the assay was 2.2%.

Reagents

- Wako Free Cholesterol C (code No. 274-47106, wako chemicals, GmbH, UK, LTD).
- Buffer solution, use as supplied
- Colour reagent, reconstituted in 75 ml of buffer solution.

Standards

- Standard Solution (supplied with kit)
- Diluent for standard solution.

Quality Control

Two controls were used. Normal control serum 1 and abnormal control serum 2 (Catalogue No. 410-00101 and No 416-00201, wako chemicals, GmbH, UK, LTD). Both controls had been reconstituted Wako 1 and Wako 2 in 5 ml distilled or deionised water.

Calculation of esterified cholesterol

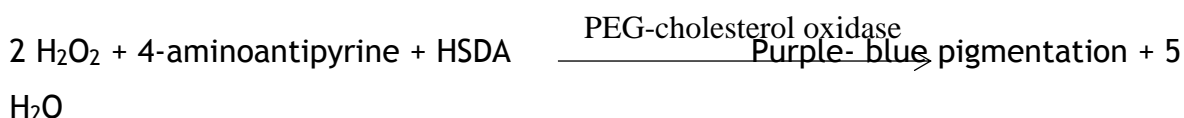
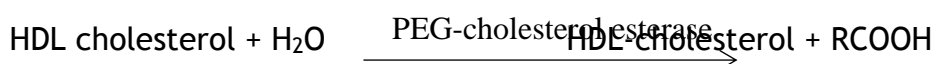
CE concentration was calculated by multiplying the difference in mass (mg.dl^{-1}) of TC and FC by 1.68 and converted to mmol.l^{-1} by dividing with 38.7.

Determination of plasma HDL cholesterol

Determinations for plasma HDL cholesterol were performed using commercially available enzymatic kits (Roche Diagnostics, Mannheim, Germany). Samples were analysed in IL600 as mentioned above.

Principle

The cholesterol concentration of HDL-cholesterol is determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with polyethylene glycol (PEG) to the amino groups. In the presence of magnesium sulphate, dextran sulphate selectively forms water-soluble complexes with LDL, VLDL and chylomicrons which are resistant to PEG- modified enzymes. Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase, in the presence of oxygen, cholesterol is oxidised by cholesterol oxidase to cholesterolone and hydrogen peroxide. The hydrogen peroxide generated reacts with 4-amino-antipyrine and N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline to form a purple-blue dye. The colour intensity of the dye is directly proportional to the cholesterol concentration and is measured photometrically at 600 nm. The equation is shown below:



(HSDA: Sodium N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline). The CV for the assay was 2.8%.

Reagents

- HDL-C Reagent 3rd generation (Cat No 04713214, Roche Diagnostics, Mannheim, Germany)
- Sodium Chloride (NaCl).

Standards

- 0.9% NaCl (used as Standard Blank).
- Roche CFAS Lipids calibrator (Cat. No.12172623, Roche Diagnostics, Mannheim, Germany) was reconstituted in 1 ml distilled water.

Quality Control

Two controls were included in the kit. Normal lipid control (LPD1) and abnormal Lipid control (LPD2)(Catalogue No LE 2661 and No LE 2663, Randox Laboratories, Crumlin, UK. Each control has been reconstituted in 3 ml distilled or deionised water.

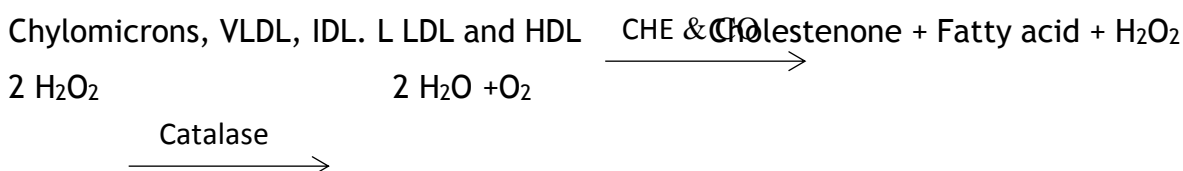
Determination of plasma small dense LDL

Determinations for plasma sdLDL cholesterol were performed using commercially available enzymatic and turbidimetric kits (Denka Seiken, Japan). Samples were analysed in ILab600 as mentioned above.

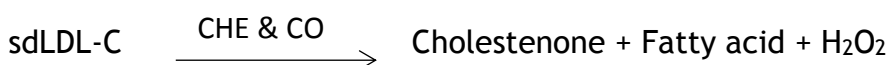
Principle

The principle of cholesterol determination is based on the following 2 steps of colorimetric reaction: of two steps; the first step removes non-sdLDL lipoproteins (chylomicrons, VLDL, IDL, L LDL and HDL) using a surfactant and sphingomyelinase in Reagent 1, where the released cholesterol is then degraded by standard enzymatic reactions; in the second step, another specific surfactant releases cholesterol only from the sdLDL particles and the catalase in Reagent 1 is inhibited by sodiumazide while the hydrogen peroxide produced from the reaction of cholesterol esterase and cholesterol oxidase results in a purple red colour with the coupler in the presence of peroxidase. The colour intensity of the dye is directly proportional to the cholesterol concentration and is measured photometrically at 550 nm. The equation is shown below:

First Step



Second Step



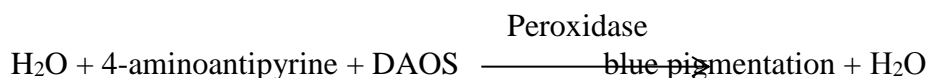
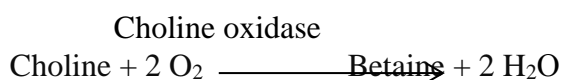
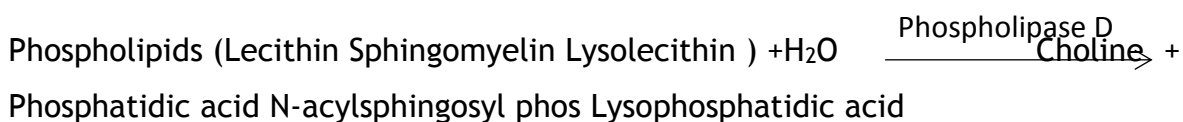
(TOOS: N-Ethyl-n-(2-hydroxy-3-sulfofopropyl)-3-methylaniline). The CV for the assay was 2.8%.

Determination of lipoprotein phospholipid

Determinations of phospholipid concentrations in lipoprotein subfractions were performed using spectrophotometric test for using available enzymatic and turbidimetric kits (Roche Diagnostics, UK). Samples were analysed in IL600 as mentioned above.

Principle

Phospholipids in serum are hydrolysed to free choline by phospholipase D. The liberated choline is subsequently oxidised with simultaneous production of hydrogen peroxide. The hydrogen peroxide, which is produced quantitatively, oxidatively couples 4- aminoantipyrine and phenol to yield a chromogen with a maximum absorbance at 570 nm. The equation is shown below:



Reagents

- Phospholipid available Kit (Cat No. MRP2 691844, Roche Diagnostics, UK).
- Bottle 1 - Buffer
- Bottle 2 - Enzyme reagent

Working reagent solution was prepared by dissolving contents of one bottle 2 in 40 ml buffer from bottle 1.

Standards

- Bottle 3 - Choline Chloride Standard Solution 300mg/dl, supplied ready to use.
- Use 0.9% NaCl as Standard Blank

Quality Control

Diluted Quality control 1:5 using 0.9% Saline (cat no 5 9020 99 10 065, Diagnostic Systems GmbH, Germany).

Determination of lipoprotein ApoE

Determinations of ApoE concentrations in lipoprotein subfractions were determined by turbidometric immunoassay using commercially available turbidimetric kits (Randox Laboratories, Crumlin, UK). Samples were analysed in IL600 as mentioned above.

Principle

Through specific antibody binding to Apolipoprotein E in plasma, insoluble aggregates are formed which cause an increase in turbidity. The level of turbidity was measured at 340 nm. The CV for the assay was 3.3%.

Reagents

- Apolipoprotein E Diagnostic Kit - Randox Apo E (Cat No LP 3864, Randox Laboratories, Crumlin, UK), used as supplied.

Standards

- Apolipoprotein calibrator (Cat No 3023, Randox Laboratories, Crumlin, UK).

Quality Control

Two controls were included in the kit. Normal lipid control (LPD1) and abnormal Lipid control (LPD3)(Catalogue No LE 2661 and No LE 2663, Randox Laboratories, Crumlin, UK. Each control has been reconstituted in 3 ml distilled or deionised water.

Determination of ApolipoproteinB Analysis

This is a turbidometric immunoassay using commercially available kits (Randox Laboratories, Crumlin, UK). Through specific antibody binding to Apolipoprotein B in plasma, insoluble aggregates are formed which cause an increase in turbidity. The level of turbidity is measured using an IL600 analyser nm and is proportional to the amount of Apo B (mg.dl^{-1}) present.

Principle

This method is based on the reaction of a sample containing human apolipoprotein-B (apoB) and specific antibodies to form an insoluble complex which can be measured turbidimetrically at 340 nm in an autoanalyser (ILab™ 600, Clinical Chemistry System, Instrumentation Laboratory, USA) . The method in **Vascular Biochemistry Lab** has been optimised for apoB measurements in lipoprotein fractions by increasing the volume of sample used in the assay from 3 μl to 12 μl (results were then multiplied by a factor of 0.25). The supplied quality control was also diluted to account for the lower concentrations of apoB. The CV of apoB in plasma was 3.8 and 2.5% and 3.2% in VLDL₁ and VLDL₂ respectively.

Reagents

Apolipoprotein B Diagnostic Kit - Randox Apo B (Cat No LP 3839), distributed by Randox Laboratories (Fridge 1 beside analyser). Use as supplied.

Standards

Calibration is not necessary as it is linked to the standard curve on SOP/LIPRES/159 (Apo B) partnered 10 coefficient 0.25

Quality Control

Two controls were included in the kit. Normal lipid control (LPD1) and abnormal Lipid control (LPD2)(Catalogue No LE 2661 and No LE 2663, Randox Laboratories, Crumlin, UK. Each control has been reconstituted in 3 ml distilled or deionised water.

LPDA made by diluting LPD1 1:5 with deionised water

Determination of Apolipoprotein C-II Analysis

This is a turbidometric immunoassay using commercially available kits (Radox Laboratories, Crumlin, UK). Through specific antibody binding to Apolipoprotein CII in the sample, insoluble aggregates are formed which cause an increase in turbidity. The level of turbidity is measured using an IL600 analyser and is proportional to the amount of Apo CII (mg.dl^{-1}) present.

Principle

This method is based on the reaction of a sample containing human ApoC-II and specific antiserum to form an insoluble complex which can be measured turbidimetrically at 340 nm. The CV for the assay was 3.3%

Reagents

Apolipoprotein CII Diagnostic Kit - Radox Apo CII (Cat No LP 3866), distributed by (Radox Laboratories. (Fridge 1 beside analyser). Used as supplied

Standard

Calibration is not necessary as it is linked to the standard curve on SOP/LIPRES/160 (Apo C2))

Partnered 6 coefficient 0.25

Quality Control

Two controls were included in the kit. Normal lipid control (LPD1) and abnormal Lipid control (LPD2)(Catalogue No LE 2661 and No LE 2663, Radox Laboratories, Crumlin, UK. Each control has been reconstituted in 3 ml distilled or deionised water.

LPDA made by diluting LPD1 1:5 with deionised water

Determination of Apolipoprotein C-III Analysis

This is a turbidometric immunoassay. Through specific antibody binding to Apolipoprotein CIII in plasma, insoluble aggregates are formed which cause an increase in turbidity. The level of turbidity is measured using an ILab600 analyser and is proportional to the amount of Apo CIII ($\text{mg} \cdot \text{dl}^{-1}$) present.

Principle

This method is based on the reaction of a sample containing human ApoC-II and specific antiserum to form an insoluble complex which can be measured turbidimetrically at 340 nm. The CV for the assay was 3.3%

Reagents

Apolipoprotein CIII Diagnostic Kit - Randox Apo CIII (Cat No LP 3865), distributed by Randox Laboratories.
Use as supplied.

Standards

- Apolipoprotein calibrator (Randox LP Cat No 3023), distributed by Randox Laboratories (Fridge 1 beside analyser). Calibrator values are lot specific.

To prepare std curve reconstitute one vial of calibrator with 1ml of distilled water at room temperature with gentle mixing, - Apo Cal .

Quality Control

Two controls were included in the kit. Normal lipid control (LPD1) and abnormal Lipid control (LPD2)(Catalogue No LE 2661 and No LE 2663, Randox Laboratories, Crumlin, UK. Each control has been reconstituted in 3 ml distilled or deionised water.

Appendix D; participants Feedback Sheet



University of Glasgow | College of Medical,
Veterinary & Life Sciences

**Title: The effects of exercise on lipoprotein
affinity for lipoprotein lipase**

**Lay title: The effects of exercise on fats in the
blood**

RESULTS FEEDBACK

Name:

DOB:

Address:

Study Start Date:

Study End Date:

Principal Researcher:

Tel: 0141 2329494

E-mail: k.ghafouri.1@research.gla.ac.uk

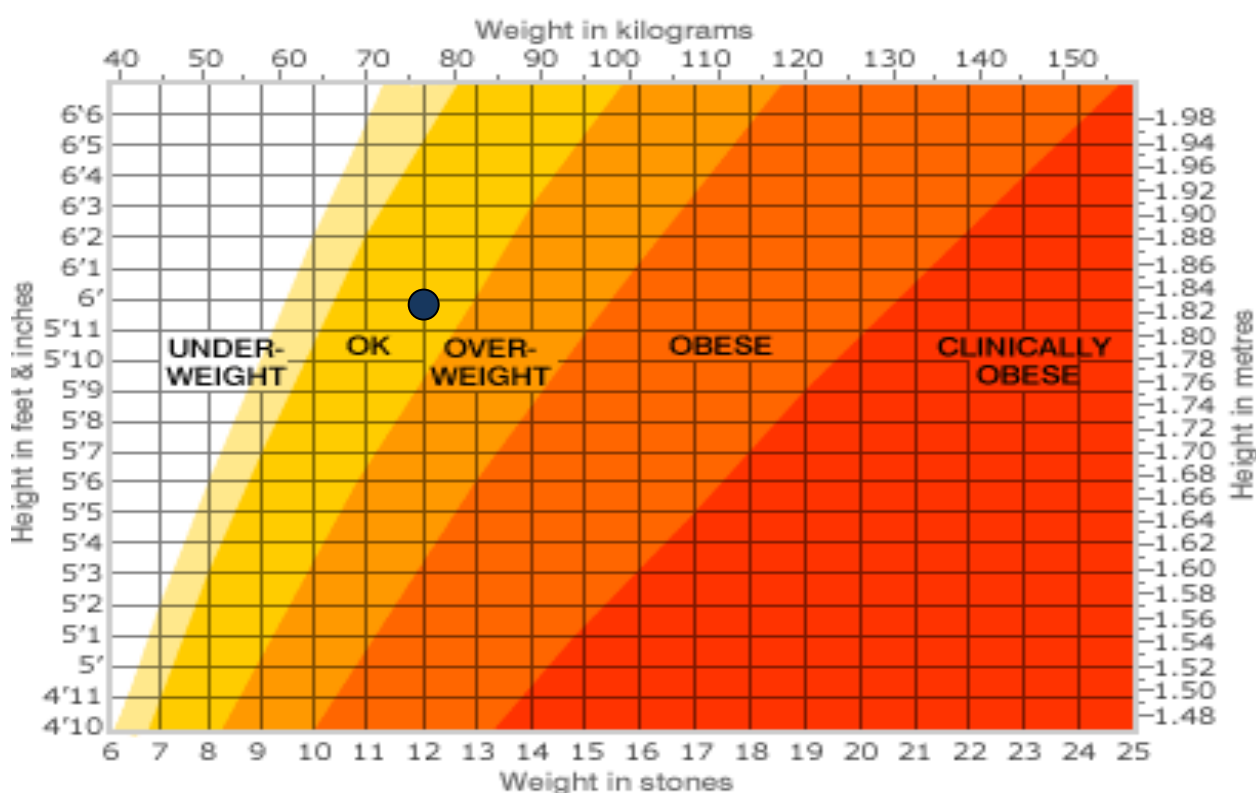
Body Composition Measurements

1. Height and Weight

Height and body mass are widely used to measure body fatness. An index called the '**Body mass index**' or '**BMI**' can be used to determine whether somebody is the correct weight for his or her height. Usually, a BMI value of 20 to 25 is normal, 25 to 30 is overweight, and 30+ is classed as obese. (BMI is calculated by dividing body mass in kg by height in metres squared, i.e. kg/m^2). However, this index is of limited value, as it does not take into account an individual's build and does not distinguish between fat and muscle mass. (In fact a number of athletes would be classed as overweight by this index, due to their large muscle mass.)

Your height **xx** **cm** **xx ft xx inch**
Your weight: **xx** **kg** (**xx stn xx lbs**)
Your body mass index (BMI): **xx** **kg/m²**

The following graph shows the ideal body mass based on BMI and optimized for men. According to your height, your body mass should be between about xx-xx stones.



2. Skinfolds

When deciding how fat somebody is, it is more useful to consider body fat levels rather than weight. A body fat estimation was calculated from measurements of the fat layer under the skin at four sites (biceps (front of the arm), triceps (back of the arm), subscapular (under the shoulder blade) and suprailiac (above the hip)). This was expressed as a percentage of total body mass (e.g. an 80kg man with 25% body fat would be carrying 20kg of fat). High percentages of body fat are linked with a number of diseases including heart disease and diabetes. For an adult male, body fat should ideally be between about 15-25%.

Your Biceps: **xx** **mm**
Your Triceps: **xx** **mm**
Your Subscapular: **xx** **mm**
Your Suprailiac: **xx** **mm**
Your Percentage Body Fat (%BF): **xx** %

3. Waist Circumference

The waist circumference is perhaps of greater importance than BMI or % body fat in determining risk of metabolic disorders such as diabetes and heart disease. This is because abdominal fat is thought to be in a position anatomically (i.e. near to the liver and other internal organs) where it could potentially cause a lot of harm. A waist measurement of greater than 100 cm (39.5 in) may increase the risk of certain metabolic complications.

Your Waist Circumference (WC): **xx** **cm** (**xx** inch)

Health Screening Results

(A copy of your blood results has been sent to your GP.)

1. Blood Pressure

Your Blood Pressure: **xx** mm Hg

A blood pressure of **xx mm Hg** is considered (**low, normal or high**). The systolic pressure (**xx mm Hg**) indicates how hard the heart is working and the force that is blood exerts when blood is pumped from the heart. The diastolic pressure (**xx mm Hg**) tells us what resistance there is to blood flow and therefore how easily blood flows through the blood vessels.

2. Fasting Glucose

Fasting glucose level is used to determine whether you have diabetes or not. The normal range of fasting glucose is 3.5-5.5 mmol/l and a value of greater than 7 mmol/l suggests diabetes.

Your Fasting Glucose: **xx** mmol/l (which is **low, normal or high**)

3. Blood Lipid Measurements

Lipids, or fats, are normal constituents of the 'watery' part of the blood, known as plasma. They are present in a number of forms, but in this study we are most interested in cholesterol and triglycerides.

Cholesterol is a fundamental component of all the cells in our bodies and is essential for the normal functioning of the body. Part of the cholesterol in plasma comes from the liver, where it is made, the remainder being absorbed from the diet. Ideally, the level of cholesterol in the plasma should not exceed 5.0 mmol/l.

Your Cholesterol: **xx** mmol/l (which is **low, normal or high**)

High-density lipoprotein cholesterol or **HDL cholesterol** is commonly referred to as 'good cholesterol'. These particles are responsible for transporting excess cholesterol away from the cells to the liver where it can be disposed of safely. High levels of HDL cholesterol are associated with a **reduced** risk of heart disease. It is desirable for your HDL concentration to be **above** 1.0 mmol/l.

Your HDL Cholesterol: xx mmol/l (which is **low, normal or high**)

Low-density lipoprotein cholesterol or LDL cholesterol, on the other hand, is commonly referred to as 'bad cholesterol'. These particles are responsible for transporting cholesterol from the liver to all cells of the body. This is essential as cholesterol is an integral component of every cell. However, high levels of LDL cholesterol will result in the deposition of cholesterol inside the arteries which is associated with an **increased** risk of heart disease. It is desirable for your LDL cholesterol to be **below** 3.0 mmol/l.

Your LDL Cholesterol: xx mmol/l (which is **low, normal or high**)

Cholesterol-to-HDL (Chol/HDL) ratio is the ratio of 'bad' to 'good' cholesterol. Ideally, it should be less than 5.0.

Your Chol/HDL ratio: xx

Triglyceride (TG) is an important source of energy and like cholesterol is both made by the liver and absorbed from the diet. Concentrations of TG in plasma rise after eating a fatty meal, but after an overnight fast it is desirable for concentrations to be below 2.3 mmol/l.

Your Triglycerides: xx mmol/l (which is **low, normal or high**)

Many people living in Western societies have elevated levels of cholesterol and TG. Comparisons of the incidence of coronary heart disease in different societies indicate that high levels of lipids are associated with increased risk of heart disease. However, it is important to realise that an elevated blood lipid concentration is only one of several well-documented risk factors for coronary heart disease, others include family history, high blood pressure, diabetes, obesity and smoking. In most circumstances it is only when a person exhibits several of these risk factors that there is a major cause for concern.

Fitness Analysis

1. Predicted Maximum Heart Rate (HR_{max})

This is the maximum number of beats that your heart can produce in a minute (beat per minute or BPM). It is calculated using the equation of $(220 - \text{your age})$. Common use for this value is for developing fitness programs and prescribing exercise intensities.

Your Predicted Maximum Heart Rate (HR_{max}): **xx** **BPM**

2. Predicted Maximal Oxygen Uptake (VO_{2max})

Your maximal oxygen uptake or VO_{2max} is a measure of your body's ability to use oxygen and is one measure of endurance fitness. The point at which you perform exercise at increasing intensity without consuming more oxygen is considered your VO_{2max} and is the point at which exercise can no longer normally be sustained. The major determinant of VO₂ max is genetics, or how well you chose your parents. Another big factor is how heavy you are, as the value is expressed per kg body mass (your VO_{2max} will increase if you lose weight). Perhaps a better measure of endurance fitness is the ability to sustain a reasonable percentage of VO_{2max} for a prolonged period. This, quite clearly, is something that you can manage well!

Your Maximal Oxygen Uptake (VO_{2max}): **xx** **ml O₂/kg/min**

The fitness classifications for *your* age group and gender are shown below:

Fitness category	VO_{2max} (ml O₂/kg/min)
Excellent	44+
Good	35-43
Average	29-34
Below average	23-28
Poor	<23

3. Energy Expenditure

The body requires energy for every physical activity which is dependent on the duration and type of activity and the body's age and gender. Energy is measured in calories (cal) and is obtained from the body stores or the food we eat, namely carbohydrates, fat and protein. The longer and harder the exercise is, the more calories you burn in order to sustain it. In order to lose 1 pound of fat, you need to burn 3500 kcal (7700 kcal for 1 kg). For the 90-minute brisk walking session you performed:

Your Energy Expenditure: **xx** **kcal**

4. Exercise Recommendations

We asked you in this study to walk for 90 minutes at about 50% of your VO_{2max} to maximise the potential effects of exercise on your body's fat metabolism. This would allow us to see the effects of exercise on fat metabolism more clearly. In practice, you don't have to perform such long exercise sessions to get a beneficial effect. We would recommend that you perform a total of 30 minutes of moderate intensity exercise (e.g. walking, gardening, golf, tennis, cycling, swimming etc.) on most, preferably all days of the week. This 30 minutes does not need to be continuous - you could split it up into a number of shorter exercise periods (each of at least 10 minutes). In addition, everyday activities such as walking to the shops can all count towards your daily exercise. This amount of exercise is the ideal, but taking any exercise at all will be beneficial. *We recommend* you to exercise at an exercise intensity of 50-70% of your VO_{2max} . **For you, this would be at a heart rate range of xx BPM.**

∞ Thank you for your time and participation
∞

Appendix E; Statistician Report about statistical test used in Chapter 4

Centre of Statistics and Development

15 Queen Square, Leeds LS2 8AJ

Phone: (44) 773-780 3857

Fax: (44) 113-281 6770

Email: info@statsd.co.uk

Website: www.statsd.co.uk



Statistical review of PhD thesis

"Effect of Exercise, Diet and Ethnicity on postprandial metabolism"

In particular **Chapter 4**

"Effect of Exercise on the Affinity of Lipoproteins for Lipoprotein Lipase"

Submitted by **Khloud Ghafouri**

The statistical objectives of this study are to compare two or more groups and to provide estimates and confidence intervals for the size of the effect as well as p-values.

Many statistical tools assume that the variable should be normally distributed. A significant violation of the assumption of normality can seriously result in the chances of the researcher committing either a Type I or II error (depending on the nature of the analysis and the non-normality). Thus, one reason (although not the only reason) researchers using data transformations is improving the normality of variables. Statistical tests are often more meaningful and accurate if outcomes are transformed, and a common choice for transforming variables is to apply logarithmic transformation. The advantage of transformations is the increase in statistical power from using parametric statistics over nonparametric statistics. Outcome means may be directly transformed back to the original scale for all these transformation, allowing outcome differences on the transformed scale to be interpreted as ratios on the original scale.

I confirm that the statistical analysis used in this thesis is correct and no further statistical work needs to be done.

Many Thanks

Dr Farag Shuweihdi (PhD in Statistics)

Director of CSD